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(54) Title: AN HSP70 PEPTIDE STIMULATING NATURAL KILLER (NK) CELL ACTIVITY AND USES THEREOF

(57) Abstract: The present invention relates to an immunostimulatory peptide derived from an Hsp70 protein and peptides comprising said immunostimulatory peptide. Furthermore the present invention pertains to polynucleotides encoding said peptide, vectors comprising said polynucleotides, fusion (poly)peptides comprising said peptide and compositions comprising said peptide. In addition the present invention relates to the use of said peptide, polynucleotide, vector or fusion (poly)peptide, for the preparation of pharmaceutical compositions for the treatment of diseases and for the stimulation of natural killer cell (NK cell) activity.

An Hsp70 peptide stimulating Natural Killer (NK) c ll activity and uses thereof

The present invention relates to an immunostimulatory peptide derived from an Hsp70 protein and peptides comprising said immunostimulatory peptide. Furthermore the present invention pertains to polynucleotides encoding said peptide, vectors comprising said polynucleotides, fusion (poly)peptides comprising said peptide and compositions comprising said peptide. In addition the present invention relates to the use of said peptide, polynucleotide, vector or fusion (poly)peptide, for the preparation of pharmaceutical compositions for the treatment of diseases and for the stimulation of natural killer cell (NK cell) activity.

Several documents are cited throughout the text of this specification. The disclosure content of each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) is herewith incorporated herein by reference.

Heat shock proteins (HSP) are highly conserved proteins that are inducible by a variety of stressful stimuli and by physiological processes including cell differentiation and development (Lindquist and Craig. 1988). Intracellular HSP function as molecular chaperones, they are involved in protein folding, transport, antigen processing and presentation (DeNagel and Pierce, 1992; Hartl, 1996). HSP with a molecular weight of 70 and 90 kDa also have been shown to function as carrier proteins for immunogenic tumor-derived peptides that induce a T cell mediated immune response against cancer (Tamura et al., 1997; Schild et al., 1999; Srivastava et al., 1998). Antigen presenting cells are key for the receptor mediated uptake of HSP-peptide complexes (Arnold-Schild et al., 1999) Several groups reported an unusual plasma membrane localization of HSP on tumor cells (Altmeyer et al., 1996; Ferrarini et al., 1992; Piselli et al., 1995; Tamura et al., 1993). The inventors were the first who demonstrated that NK cells also have to be considered as relevant effector cells for the recognition of membrane-bound Hsp70 on tumor cells (Multhoff et al., 1995a, 1995b; Multhoff et al., 1997; Botzler et al., 1996a, 1996b). With respect to these findings and due to the fact that normal cells lack

expression of Hsp70, the inducible member of the Hsp70 group on the plasma membrane, one might speculate that Hsp70 acts as a tumor-selective recognition structure for NK cells. Antibody blocking studies revealed that Hsp70 is a relevant recognition structure for transiently plastic adherent NK cells (Multhoff et al., 1995a, 1995b; Multhoff et al., 1997; Botzler et al., 1998). Although several antibodies detect membrane-bound Hsp70 on tumor cells, only the mAb RPN1197 was able to block the cytolytic activity of NK cells (Multhoff et al. 1995a).
It was recently demonstrated that proliferation and cytolytic activity of NK cells against Hsp70-expressing tumor cells could be stimulated with recombinant Hsp70 protein but not with Hsc70 or DnaK (Multhoff et al. 1999). As target cells for the cytolytic activity of NK cells the tumor sublines CX+ and CX- with an identical MHC and adhesion molecule expression pattern that differ with respect to the capacity to express Hsp70 on the plasma membrane, were used (Multhoff et al. 1997). It was furthermore demonstrated that not only intact Hsp70 protein but also the C-terminal domain of Hsp70hom activates NK cells. Hsp70hom, a testis specific member of the Hsp70 family, is 94% homologous to the C-terminal domain of Hsp70.

For the production of a (poly)peptide and its formulation in pharmaceutical compositions it is generally desirable to reduce its size as far as is reasonable with respect to its biological activity. A reduction in size and complexity increases the yield of a recombinantly expressed peptide and generally increases its chemical stability. When producing a peptide synthetically, yield and reliability of the process are also increased with a peptide of reduced size whereas production cost is minimized.

Therefore the technical problem underlying the present invention was to provide a small, easily obtainable molecule with immunostimulatory activity which can be produced synthetically or recombinantly in large amounts and at low cost. The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention provides a peptide comprising or having the amino acid sequence TKDNNLLGRFELXG wherein X is T or S, wherein S is

preferred (also throughout the further embodiments recited in this specification). The invention also encompasses fragments or derivatives of said peptide which will be explained further below.

The tem "peptide" as used herein denotes amino acid sequences comprising 30 or less amino acids.

It has surprisingly been found that a peptide comprising or having the sequence TKDNNLLGRFELXG, wherein X is T or S, wherein S is preferred, is sufficient for the stimulation of NK cell activation. At least for in vitro purposes, an advantageous final concentration of the peptides has been found to be in the range of 0.2 to 2.5 µg/ml.

From the data available from the prior art it was not deducible that a peptide structure that encompasses hardly more than an antibody binding epitope might trigger such a complex cascade of events. In contrast, it was generally believed that a peptide alone is not sufficient to trigger T cell activation. The size of the structure that was found sufficient to trigger such events excludes that cross-linking events are involved in the stimulation of NK activity by the underlying mechanism which, again, is surprising in view of the general beliefs of the prior art.

Comprised by the invention are also fragments and derivatives of the peptide of the invention wherein the derivatives have a different amino acid sequence which deviates from that of the peptide of the invention by substitution, insertion, deletion, duplication, inversion etc. provided that said fragments or derivatives stimulate NK cell activation. It is most preferred that in these peptides, amino acids TKDN in positions 450 to 453 (of Hsp70) R in position 458 and S in position 462 are retained. Said derivatives or fragments may be further derivatized by e.g. peptidomimetics as will be outlined below. They may also form part of a fusion protein as described below. The derivatives and fragments that are composed by the present invention may be tested, without undue burden for functionality and medical usefulness as described throughout this specification and as described in particular in the appended examples. The derivatives or fragments preferably have the length of at least 13 amino acids and are preferably not longer than 30 amino acids, more

preferably not longer than 20 amino acids.

The present invention also relates a peptide as defined supra comprising or having the amino acid sequence EGERAMTKDNNLLGRFELXG wherein X is T or S. The invention also encompasses fragments or derivatives of said peptide which have the activation function and can be selected as described supra.

The peptide of the present invention may be linked to other (poly)peptide sequences or may be part of (a) fusion (poly)peptide(s). Such fusion (poly)peptides/ fusionproteins may be engineered to improve the characteristics of said fragments, derivatives or variants. For example, further amino acids may be added to improve stability and/or persistence during purification, handling or storage processes or to improve stability, half-life and/or persistence in vitro and in host organisms and/or patients. Furthermore, the peptide of the invention may be fused to other proteins or peptides which play a role in immune responses or in their potential treatment. Within the scope of the present invention are also molecules which comprise the peptide of the invention which are linked to marker molecules and/or marker amino acid sequences. Such sequences comprise but are not limited to peptide-tags, histidine-tags, fluorescence molecules, GFP, FLAG and GST.

Therefore, the invention furthermore relates to a fusion (poly)peptide comprising the peptide of the invention.

The invention also relates to a polynucleotide encoding the peptide of the invention or said fusion (poly)peptide comprising the peptide of the invention.

The polynucleotide as employed in accordance with this invention and encoding the above-described peptide may be, e.g., DNA, cDNA, RNA or synthetically produced DNA or RNA or a recombinantly produced chimeric nucleic acid molecule comprising any of those polynucleotides either alone or in combination.

In a further embodiment, the invention relates to a nucleic acid molecule of at least 15 nucleotides in length hybridizing with a polynucleotide as described above or with

a complementary strand thereof. Specific hybridization occurs preferably under stringent conditions and implies no or very little cross-hybridization with nucleotide sequences encoding no or substantially different peptides. Such nucleic acid molecules may be used as probes and/or for the control of gene expression. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary in length. Preferred are nucleic acid probes of 17 to 35 nucleotides in length. Of course, it may also be appropriate to use nucleic acids of up to 100 and more nucleotides in length. Therefore, the polynucleotides encoding the peptide of the invention may be used as nucleic acid probes to identify nucleic acids molecules encoding (poly)peptides different from HSP70 comprising the peptide of the invention. Such (poly)peptides may be useful tools for investigating different pathways which may be involved in the activation of NK cells. Said nucleic acid probes are also useful for various pharmaceutical and/or diagnostic applications. On the one hand, they may be used as PCR primers for the amplification of polynucleotides encoding the peptide of the invention. In this context they may serve as useful diagnostic tools to determine e.g. the expression level of (poly)peptides comprising the peptide of the invention thereby assessing or predicting the status of NK cell activity. Nucleic acid molecules employed in this preferred embodiment of the invention which are complementary to a polynucleotide as described above may also be used for repression of expression of a gene comprising such a polynucleotide, for example due to an antisense or triple helix effect or for the construction of appropriate ribozymes (see, e.g., EP-A1 0 291 533, EP A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a gene comprising a polynucleotide as described herein above. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460. Standard methods relating to antisense technology have also been described (Melani, Cancer Res. (1991), 2897-2901). Said antisense or triple helix effect as well as the construction of relevant ribozymes is/are partially useful in pharmaceutical compositions to be employed for the suppression of NK-cell activity, e.g., in autoimmune or inflammatory diseases, viral infections, sepsis, etc. Furthermore, the person skilled in the art is well aware that it is also possible to label such a nucleic acid probe with an appropriate marker for

specific (Inter alia, diagnostic) applications, such as for the detection of the presence of a polynucleotide as described herein above in a sample derived from an organism.

The above described nucleic acid molecules may either be DNA or RNA or a hybrid thereof. Furthermore, said nucleic acid molecule may either contain, for example, thioester bonds and/or nucleotide analogues, commonly used in oligonucleotide anti-sense approaches. Said modifications may be useful for the stabilization of the nucleic acid molecule against endo- and/or exonucleases in the cell. Said nucleic acid molecules may be transcribed by an appropriate vector containing a chimeric gene which allows for the transcription of said nucleic acid molecule in the cell.

With respect to the nucleotide sequences characterized above, the term "hybridizing" in this context is understood as referring to conventional hybridization conditions, preferably such as hybridization in 50%formamide/6×SSC/0.1%SDS/100μg/ml ssDNA, in which temperatures for hybridization are above 37°C and temperatures for washing in 0.1xSSC/0.1%SDS are above 55°C. Most preferably, the term "hybridizing" refers to stringent hybridization conditions, for example such as described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. Stringent hybridization conditions include hybridization at 65°C in 0.2 x SSC, 0.1% SDS.

In a further embodiment the invention pertains to a vector comprising the polynucleotide encoding the peptide of the invention.

Many suitable vectors are known to those skilled in molecular biology, the choice of which would depend on the function desired and include plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989), (1994). Alternatively, the polynucleotides and vectors of the invention can be reconstituted into liposomes for

delivery to target cells. As discussed in further details below, a cloning vector was used to isolate individual sequences of DNA. Relevant sequences can be transferred into expression vectors where expression of a particular polypeptide is required. Typical cloning vectors include pBscpt sk, pGEM, pUC9, pBR322 and pGBT9. Typical expression vectors include pTRE, pCAL-n-EK, pESP-1, pOP13CAT.

Hence, in a preferred embodiment of the present invention the polynucleotides encoding the peptide of the invention either alone or present in a vector are linked to control sequences which allow the expression of the polynucleotide in prokaryotic and/or eukaryotic cells.

The term "control sequence" refers to regulatory DNA sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, control sequences generally include promoter, ribosomal binding site, and terminators. In eukaryotes generally control sequences include promoters, terminators and, in some instances, enhancers, transactivators or transcription factors. The term "control sequence" is intended to include, at a minimum, all components the presence of which are necessary for expression, and may also include additional advantageous components.

The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is obvious for a skilled person that double-stranded nucleic acid is preferably used.

Thus, the vector of the invention is preferably an expression vector. An "expression vector" is a construct that can be used to transform a selected host cell and provides for expression of a coding sequence in the selected host. Expression vectors can for instance be cloning vectors, binary vectors or integrating vectors. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotic and/or eukaryotic cells are well known to those skilled in the art. In the case of eukaryotic cells they

comprise normally promoters ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the P_L, lac, trp or tac promoter in E. coli, and examples of regulatory elements permitting expression in eukaryotic host cells are the AOX1 or GAL1 promoter in yeast or the CMV-, SV40-, RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), pSPORT1 (GIBCO BRL). An alternative expression system which could be used to express a cell cycle interacting protein is an insect system. In one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in Spodoptera frugiperda cells or in Trichoplusia larvae. The coding sequence of a nucleic acid molecule of the invention may be cloned into a nonessential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of said coding sequence will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses are then used to infect S. frugiperda cells or Trichoplusia larvae in which the protein of the invention is expressed (Smith et al., 1983; Engelhard et al., 1994).

To obtain agents derived from the peptide of the present invention with a further increased stability and to improve the uptake via the gastrointestinal tract or other routes different from a parental application like e.g. skin or lung peptidomimetics may be utilized to design pseudopeptide analogues. A computer redesign of the structure of the peptide of the invention can be performed using appropriate computer programs (Olszewski et al., 1996; Hoffman et al., 1995). In particular, the appropriate programs can be used for the identification of interactive sites of the peptide and, if present, its receptor or other interacting proteins by computer assistant searches for complementary peptide sequences (Fassina, 1994). Further appropriate computer systems for the design of protein and peptides are described in the prior art, for example in Berry et al. (1994), Wodak et al., (1987) or Pabo et al. (1986). The results obtained from the above-described computer analysis can be

used for, e.g., the preparation by peptidomimetics of the peptide of the invention. Such pseudopeptide analogues of the natural amino acid sequence of the peptide may very efficiently mimic the parent protein (Benkirane et al., 1996). For example, incorporation of easily available achiral Ω-amino acid residues into the peptide of the invention results in the substitution of amide bonds by polymethylene units of an aliphatic chain, thereby providing a convenient strategy for constructing a peptide by peptidomimetics (Banerjee et al., 1996). Superactive peptidomimetic analogues of small peptide hormones in other systems are described in the prior art (Zhang et al., 1996). Appropriate peptidomimetics of the peptide of the present invention can also be identified by the synthesis of peptidomimetic combinatorial libraries through successive amide alkylation and testing the resulting compounds, e.g., for their immunological properties. Methods for the generation and use of peptidomimetic combinatorial libraries are described in the prior art, for example in Ostresh et al. (1996), and Dorner et al. (1996).
Furthermore, a three-dimensional and/or crystallographic structure of the peptide of the invention can be used for the design of peptidomimetic inhibitors of the biological activity of the protein of the invention (Rose et al., 1996; Rutenber et al., 1996).

The invention therefore also relates to a method of refining the peptide of the invention comprising (a) modeling said peptide by peptidomimetics and (b) chemically synthesizing the modeled peptide. A most suitable starting point for modeling by peptidomimetics is to test libraries of peptides of different lengths and sequences for stimulating NK-cell activation. By determining where (i.e. to which amino acid residues of which protein) the peptide of the invention binds to an NK-cell the crucial amino acids for binding within the peptide of the invention can be identified. In following steps, the peptide of the invention can be optimized by chemical modification so that a more efficient stimulation of NK-cell activation is achieved. Other putative NK cell activators can be modeled in the same way.

In another embodiment the present invention further relates to a composition comprising said peptide or a fragment or derivative thereof the polynucleotide encoding said peptide or a fragment or derivative thereof, a vector comprising said polynucleotide or a fusion (poly)peptide comprising said peptide or a fragment or

derivative thereof and a pharmaceutically acceptable carrier and/or diluent.

The term "composition" as used herein also encompasses medical products and medical adjuvants and vaccines.

According to the invention, medical products are all substances or preparations used individually or in combination with each other of substances, or other subject-matters which, according to the producer, are meant to be applied to humans due to their functions for the purpose of detecting, preventing, monitoring, treating or alleviating diseases and whose main effect in or on the human body is achieved neither by pharmalogically or immunologically effective preparations nor by a metabolism whose effectiveness may well be supported by such preparations.

According to the invention, medical adjuvants are such substances which are used for the production (as active ingredients) of pharmaceutical preparations or compositions.

In a preferred embodiment the composition is a pharmaceutical composition.

Said pharmaceutical composition advantageously also comprises a pharmaceutically acceptable carrier and/or diluent. It is additionally preferred that the pharmaceutical composition recited here or produced in accordance with further embodiments of this invention further comprises IL-2 and/or IL-18 in a suitable dose, preferably in a concentration of 1 ng to 1 µg/ml.

The term pharmaceutically acceptable carrier and/or diluent generally denotes vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are sterile distilled water, physiological saline, Ringer's solutions, dextrose solution, Hank's solution, RPMI 1640 Medium and phosphate buffered saline (PBS). In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like. A therapeutically effective dose refers to that amount of protein or its antibodies, antagonists, or inhibitors which ameliorate the symptoms or condition. Therapeutic

efficacy and toxicity of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50.

Further examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. A typical dose can be, for example, in the range of 0.001 to 1000 µg (or of nucleic acid for expression or for inhibition of expression in this range); however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment.

The compositions comprising, the peptide, compound drug, pro-drug or pharmaceutically acceptable salts thereof may conveniently be administered by any of the routes conventionally used for drug administration, for instance, orally, topically, parenterally or by inhalation. Acceptable salts comprise acetate, methylester, HCL, sulfate, chloride and the like. The drugs may be administered in conventional dosage forms prepared by combining the drugs with standard pharmaceutical carriers according to conventional procedures. The drugs and pro-drugs identified and obtained in accordance with the present invention may also be

administered in conventional dosages in combination with a known, second therapeutically active compound. Such therapeutically active compounds comprise, for example, those mentioned above. These procedures may involve mixing, granulating and compressing or dissolving the ingredients as appropriate to the desired preparation. It will be appreciated that the form and character of the pharmaceutically acceptable character or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. The pharmaceutical carrier employed may be, for example, either a solid or liquid. Examplary of solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, stearic acid and the like. Exemplary of liquid carriers are phosphate buffered saline solution, syrup, oil such as peanut oil and olive oil, water, enulsions, various types of wetting agents, sterile solutions and the like. Similarly, the carrier or diluent may include time delay material well known to the art, such as glyceryl mono-stearate or glyceryl distearate alone or with a wax.

A wide variety of pharmaceutical forms can be employed. Thus, if a solid carrier is used, the preparation can be tableted, placed in a hard gelatin capsule in powder or pellet form or in the form of a troche or lozenge. The amount of solid carrier will vary widely but preferably will be from about 25 mg to about 1 g. When a liquid carrier is used, the preparation will be in the form of a syrup, emulsion, soft gelatin capsule, sterile injectable liquid such as an ampule or nonaqueaous liquid suspension.

The composition may be administered topically, that is by non-systemic administration. This includes the application externally to the epidermis or the buccal cavity and the instillation of such a compound into the ear, eye and nose, such that compound does not significantly enter the blood stream. In contrast, systemic administration refers to oral, intravenous, intraperitoneal and intramuscular administration.

Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin to the site of inflammation such as liniments, lotions, creams, ointments or pastes, and drops suitable for administration to the eye, ear or nose. The active ingredient may comprise, for

topical administration, from 0.001% to 10% w/w, for instance from 1% to 2% by weight of the formulation. It may however comprise as much as 10% w/w but preferably will comprise less than 5% w/w, more preferably from 0.1% to 1% w/w of the formulation.

Lotions according to the present invention include those suitable for application to the skin or eye which are suitable, for example, for use in UV protection. An eye lotion may comprise a sterile aqueous solution optionally containing a bactericide and may be prepared by methods similar to those for the preparation of drops. Lotions or liniments for application to the skin may also include an agent to hasten drying and to cool the skin, such as an alcohol or acetone, and/or a moisturizer such as glycerol or an oil such as castor oil or arachis oil.

Creams, ointments or pastes according to the present invention are semi-solid formulations of the active ingredient for external application. They may be made by mixing the active ingredient in finely-divided or powdered form, alone or in solution or suspension in an aqueous or non-aqueous fluid, with the aid of suitable machinery, with a greasy or non-greasy base. The base may comprise hydrocarbons such as hard, soft or liquid paraffin, glycerol, beeswax, a metallic soap; a mucilage; an oil of natural origin such as almond, corn, arachis, castor or olive oil; wool fat or its derivatives or a fatty acid such as steric or oleic acid together with an alcohol such as propylene glycol or a macrogel. The formulation may incorporate any suitable surface active agent such as an anionic, cationic or non-ionic surfactant such as a sorbitan ester or a polyoxyethylene derivative thereof. Suspending agents such as natural gums, cellulose derivatives or inorganic materials such as silicaceous silicas, and other ingredients such as lanolin, may also be included.

Drops according to the present invention may comprise sterile aqueous or oily solutions or suspensions and may be prepared by dissolving the active ingredient in a suitable aqueous solution of a bactericidal and/or fungicidal agent and/or any other suitable preservative, and preferably including a surface active agent. The resulting solution may then be clarified by filtration, transferred to a suitable container which is then sealed and sterilized by autoclaving or maintaining at 98-100°C for half an hour. Alternatively, the solution may be sterilized by filtration and transferred to the container by an aseptic technique. Examples of bactericidal and

fungicidal agents suitable for inclusion in the drops are phenylmercuric nitrate or acetate (0.002%), benzalkonium chloride (0.01%) and chlorhexidine acetate (0.01%). Suitable solvents for the preparation of an oily solution include glycerol, diluted alcohol and propylene glycol.

The composition in accordance with the present invention may be administered parenterally, that is by intravenous, intramuscular, subcutaneous intranasal, intrarectal, intravaginal or intraperitoneal administration. The subcutaneous and intramuscular forms of parenteral administration are generally preferred. Appropriate dosage forms for such administration may be prepared by conventional techniques. The composition may also be administered by inhalation, that is by intranasal and oral inhalation administration. Appropriate dosage forms for such administration, such as an aerosol formulation or a metered dose inhaler, may be prepared by conventional techniques.

For all methods of use disclosed herein for compositions according to the invention, the daily oral dosage regimen will preferably be from about 0.1 to about 80 mg/kg of total body weight, preferably from about 0.2 to 30 mg/kg, more preferably from about 0.5 mg to 15 mg. The daily parenteral dosage regimen about 0.1 to about 80 mg/kg of total body weight, preferably from about 0.2 to about 30 mg/kg, and more preferably from about 0.5 mg to 15 mg/kg. The daily topical dosage regimen will preferably be from 0.1 mg to 150 mg, administered one to four, preferably two to three times daily. The daily inhalation dosage regimen will preferably be from about 0.01 mg/kg to about 1 mg/kg per day. It will also be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of the compositions will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the particular patient being treated, and that such optimums can preferably be determined by the methods described herein. It will also be appreciated by one of skill in the art that the optimal course of treatment, i.e., the number of doses of the compositions given per day for a defined number of days, can be ascertained by those skilled in the art using conventional course of treatment determinations tests. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's

size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Progress can be monitored by periodic assessment.

In another embodiment, the DNA sequence or vector of the invention, as described above, may be directly administered to a patient in need thereof. This type of administration is generally referred to as DNA vaccination. Routes for administration of gene vaccines are well known in the art and DNA vaccination has been successfully used to elicit alloimmune, anti-tumor and antiidiotype immune responses (Tighe M. et al., 1998). Moreover, inoculation with nucleic acid molecules/DNA has been found to be protective in different modes of viral disease (Fynan, et al., 1993; Boyer, 1997; Webster, et al., 1994; Montgomery et al., 1993; Barry, 1995; Xu and Liew, 1995; Zhong et al., 1996; Luke et al., 1997; Mor, 1998; MacGregor et al., 1998).

The DNA encoding the peptide of the invention used in a pharmaceutical composition as a DNA vaccine may be formulated e.g. as neutral or salt form. Pharmaceutically acceptable salts, such as acid addition salts, and others, are known in the art. DNA vaccines are administered in dosages compatible with the method of formulation, and in such amounts that will be pharmacologically effective for prophylactic or therapeutic treatments. Preferably, the vaccine comprises an expression vector as described herein above.

Typically, vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in or suspension in liquid prior to injection also may be prepared. The preparation may be emulsified or the protein may be encapsulated in liposomes. The active immunogenic ingredients often are mixed with pharmacologically acceptable excipients which are compatible with the active ingredient. Suitable excipients include but are not limited to water, saline, dextrose, glycerol, ethanol and the like; combinations of these excipients in various amounts also may be used. The vaccine also may contain small amounts of auxiliary substances such as wetting or emulsifying reagents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. For example, such adjuvants can include aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-

isoglutamine (thr-DMP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (CGP 11687, also referred to as nor-MDP), N-acetylmuramyul-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'2'-dipalmitoyl-sn-glycero-3-hydroxphaosphoryloxy)-ethylamine (CGP 19835A, also referred to as MTP-PE), and RIBI (MPL + TDM + CWS) in a 2% squalene/Tween-80® emulsion.

The vaccines usually are administered by intravenous or intramuscular injection. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral or nasal formulations. For suppositories, traditional binders and carriers may include but are not limited to polyalkylene glycols or triglycerides. Oral formulation include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions may take the form of solutions, suspensions, tables, pills, capsules, sustained release formulations or powders and contain about 10% to about 95% of active ingredient, preferably about 25% to about 70%.

The peptide of the invention or a DNA encoding it are administered as a vaccine in a way compatible with the dosage formulation, and in such amounts as will be prophylactically and/or therapeutically effective. The quantity to be adminstered generally is in the range of about 0.2 nanograms to about 500 micrograms of peptide or DNA per dose, and depends upon the subject to be dosed, and the degree of immune-activation sought. Precise amounts of active ingredient required to be administered may also depend upon the judgment of the practitioner and may be unique to each subject. The peptide or DNA may be given in a single or multiple dose schedule. A multiple dose is one in which a primary course of vaccination may be with one to ten separate doses, followed by other doses given at subsequent time intervals required to maintain and/or to reinforce the immune response, for example, at one week to four months for a second dose, and if required by the individual, subsequent dose(s) weekly or monthly. The dosage regimen also will be determined, at least in part, by the need of the individual, and be dependent upon the practitioner's judgment. It is contemplated that the vaccine containing the immunogenic compounds of the invention may be administered in conjunction with

other immunoregulatory agents, for example, with immunoglobulins or with cytokines.

In another embodiment the method of the invention relates to a method of producing a immunostimulatory peptide comprising

(a) mutating on the DNA or amino acid level a DNA molecule encoding or peptide comprising the sequence TKDNNLLGRFELXG, wherein X is T or S, in one or more nucleotide or amino acid positions;

(b) testing the proliferative response of NK cells to stimulation with IL-2 and/or IL-18 together with the mutated peptide;

(c) comparing the proliferative response of NK cells to IL-2 and/or IL-18 together with the mutated peptide to the proliferative response to IL-2 and/or IL-18 without the mutated peptide or with a peptide having the sequence recited in (a);

(d) selecting a peptide comprising the mutated sequence or a recombinant DNA molecule coding for a peptide comprising the mutated sequence, wherein said mutated peptide displays an increase in the proliferative response as compared to IL-2 and/or IL-18 without the mutated peptide or with a sequence recited in (a) in step (c).

If nucleotides are mutated (i.e. exchanged by different naturally or not naturally occurring nucleotides), it is understood that at least one exchange should lead to an exchange on the amino acid level. It is most preferred that in these peptides, amino acids TKDN in positions 450 to 453 (of Hsp70) R in position 458 and S in position 462 are retained.

The term "proliferative response of NK cells" as used herein denotes proliferation of an NK cell population which can be determined by standard proliferation assays. Such assays comprise the measurement of incorporation of bromodeoxyuridine (BrdU) or thymidine into the cellular genome during S-phase as mentioned in the examples below. The percentage of increase should at least be 10%, preferably at least 20%, more preferably at least 50%. If the proliferative response of the mutated peptide is compared to the peptide having the sequence recited in (a), and an increase is observed, then a compound with an improved stimulatory activity may be selected.

In the method of the invention, the DNA sequence may be mutated upon which the peptide is expressed in a recombinant host such as E. coli, a mammalian or other cell. For this, the DNA sequence is usually expressed in a vector.

In a further embodiment the product is refined by computer redesign and/or peptidomimetics.

The invention also relates to a method of producing a pharmaceutical composition, medical product, medical adjuvant or vaccine comprising the step of formulating the peptide or recombinant DNA molecule selected by the method of the invention with a pharmaceutically acceptable carrier and/or diluent.

The invention further relates to a method of producing a pharmaceutical composition comprising a molecule stimulating the activation of NK cells comprising the steps of (a) modifying the peptide of the invention as a lead compound to achieve (i) modified site of action, spectrum of activity, organ specificity, and/or (ii) improved potency, and/or (iii) decreased toxicity (improved therapeutic index), and/or (iv) decreased side effects, and/or (v) modified onset of therapeutic action, duration of effect, and/or (vi) modified pharmakinetic parameters (resorption, distribution, metabolism and excretion), and/or (vii) modified physico-chemical parameters (solubility, hygroscopicity, color, taste, odor, stability, state), and/or (viii) improved general specificity, organ/tissue specificity, and/or (ix) optimized application form and route by (i) esterification of carboxyl groups, or (ii) esterification of hydroxyl groups with carbon acids, or (iii) esterification of hydroxyl groups to, e.g. phosphates, pyrophosphates or sulfates or hemi succinates, or (iv) formation of pharmaceutically acceptable salts, or (v) formation of pharmaceutically acceptable complexes, or (vi) synthesis of pharmacologically active polymers, or (vii) introduction of hydrophilic moieties, or (viii) introduction/exchange of substituents on aromates or side chains, change of substituent pattern, or (ix) modification by introduction of isosteric or bioisosteric moieties, or (x) synthesis of homologous compounds, or (xi) introduction of branched side chains, or (xii) conversion of alkyl substituents to cyclic analogues, or (xiii) derivatisation of hydroxyl group to ketales,

acetales, or (xiv) N-acetylation to amides, phenylcarbamates, or (xv) synthesis of Mannich bases, imines, or (xvi) transformation of ketones or aldehydes to Schiff's bases, oximes, acetales, ketales, enolesters, oxazolidines, thiozolidines or combinations thereof; and (b) formulating the product of said modification with a pharmaceutically acceptable carrier.

The various steps recited above are generally known in the art. They include or rely on quantitative structure-action relationship (QSAR) analyses (Kubinyi, 1993), combinatorial biochemistry, classical chemistry and others (see, for example, Holzgrabe and Bechtold, 2000).

In a another embodiment the invention relates to a method for producing a pharmaceutical composition, medical product, medical adjuvant or vaccine comprising formulating the product obtained by the above method of the invention with a pharmaceutically acceptable carrier and/or diluent.

Employing the data obtainable with the peptide of the invention, new immunostimulatory substances may be generated. The sequence of the peptide of the invention provided herein allows to design modified or alternative molecules with immunostimulatory activity. In one alternative, potential immunostimulatory substances may be tested for on natural NK cells. Those substances which, in cotreatment with IL-2 and/or IL-18, stimulate a proliferate response which is higher than the proliferative response to IL-2 and/or IL-18 alone are to be formulated into pharmaceutical compositions for the downstream development of immunostimulatory substances suitable for formulation into pharmaceutical compositions. The new stimulatory substances may be further refined by peptidomimetics or otherwise as is known in the art, i.a. depending on the specific medical purpose or route of administration or may be prepared as a fusion protein. The embodiments recited in this paragraph also belong to the present invention.

Preferred concentrations of IL-2 and/or IL-18 are in the range of 1ng - 1µg/ml.

Insofar, the invention relates also to a preferred method as stated above, wherein said product is refined by computer redesign and/or peptidomimetics.

In a still further embodiment the invention relates to the use of the peptide of the present invention the fusion (poly)peptide of the invention, the polynucleotide of the invention, the vector of the invention or the peptide refined by the method of the invention or to be formulated into a pharmaceutical composition produced by the method of the invention for the preparation of a pharmaceutical composition for the activation of NK cells.

The term "NK cells" ("natural killer cells") as used herein denotes large granular lymphocytes having CD45 surface expression and which furthermore have killer cell activity without previous stimulation. NK cells are especially characterized by epression of CD16 and/or by their susceptibility to Interleukin-2 stimulation and/or do not express CD3 and/or do not express α/β- γ/δ- T-cell receptors.

NK-cells of the present invention preferably are further characterized by the following characteristics:

- they are transiently plastic-adherent after stimulation with IL-2 in a dosage of 10 to 10.000 units, e.g. von 100 units, wherein IL-2 can be obtained from Chiron Corp.;
- Adherens can be observed 3-18 h after stimulation of previously isolated PBLs (Peripheral Blood Lymphocytes) with IL-2.;
- the NK cells express CD16dim (the mean value of fluorescence is weak);
- the NK cells express CD56 and CD57 as typical NK-markers;
- the NK-cells express CD94 (C-type lectin killer-cell- receptor);
- the NK-cells secrete IFNgamma after atcivation with Hsp70 and cytokines;
- the NK-cells can be activated (growth and cytotoxic activity) by Hsp70 (purified protein);
- they are not dependent on a patients MHC-type.

In the present invention different NK-cell populations can be applied. It is, however, required that the NK-cells as characterized herein can be activated by the peptide of the invention. It is possible to use isolated NK-cells but mixtures containing different

cell types like peripheral blood mononuclear cells (PBMC) also comprising NK cells can be applied too.

In a preferred embodiment said activation comprises the activation of the proliferation of NK cells and/or the cytolytic activity of NK cells.

In a final embodiment the invention relates to the use of the peptide of the invention the fusion (poly)peptide of the invention, the polynucleotide of the invention, the vector of the invention or the peptide refined by the method of the invention or to be formulated into a pharmaceutical composition produced by the method of the invention for the preparation of a pharmaceutical composition in immunotherapy and/or for the treatment of diseases selected from carcinomas of lung, colorectum, pancreas, larynx, stomach, peripheral and central nervous system, other carcinomas, sarcomas, chronic myeloic leukemia (CML), acute myeloic leukemia (AML), acute lymphatic leukemia (ALL), non Hodgkin Lymphoma (NHL), myeloproliferative syndrome (MPS), myelodysplastic syndrome (MDS), plasmocytoma, other leukemias, other malignant diseases, wherein Hsp70 is present on the surface of malignant cells, an infection with an HI virus, other viral or bacterial infections wherein Hsp70 is present on the surface of infected cells, rheumatoid arthritis, lupus erythematodes, other autoimmune diseases, asthma bronchiale, or other inflammatory diseases.

The figures show:

Figure 1.

Comparison of the proliferative response of NK cells against Hsp70 peptides. NK cells were stimulated either with IL-2 (100 IU/ml) alone or with IL-2 plus peptides NLL (8-mer, Figure 1a), TKD (14-mer, Figure 1b) and GIPP (13 mer, Figure 1c) at concentrations 0.02, 0.2, 2, 4 and 8 µg/ml. As a positive control NK cells were stimulated with rHsp70 protein at a concentration of 10 µg/ml. The proliferative response of NK cells was measured 72 h after peptide incubation and an 18 h incubation period with ^{3}H thymidine (1 µCi/ml). Stimulation with different peptides at a concentration of 2µg peptide per ml is shown in Figure 1d. In Figure 1e stimulation

with TKD in a more refined scale between 0.1 and 3 µg is shown. Figure 1f shows stimulation with various peptides. Values are given as the means of four independent experiments +/- SD.

Figure 2.

Cytolytic activity of NK cells stimulated with IL-2 alone or with IL-2 plus the 14-mer peptide TKD or with IL-2 plus the 8-mer peptide NLL. Cytolytic activity of NK cells either stimulated with low dose IL-2 (100 IU/ml) alone or with IL-2 plus the 14-mer peptide TKD (2 µg/ml) or with IL-2 plus the 8-mer peptide NLL (2 µg/ml) for 4 days. As target cells ^{51}Cr labeled CX+ (Figure 2a) and CX- (Figure 2b) tumor cells, that differ with respect to their capability to express Hsp70 on the plasma membrane, were used. The results are expressed as the percentage of specific lysis at varying E:T ratios ranging from 2:1 up to 40:1. The percentage spontaneous release for each tumor target cell line was less than 20%. A phenotypic characterization of the NK cells reveals the following: NK cells plus TKD: CD3, 6%; CD16/56, 79%; NK cells plus NLL: CD3, 8%; CD16/56 80%; NK cells: CD3, 5%; CD16/56: 81%.

The data represent one representative experiment out of three.

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

Example 1

Epitope mapping analysis of the Hsp70 specific mAb

Previously, we showed an unusual plasma membrane localization of Hsp70 selectively on tumor cells with the Hsp70 specific mAb RPN1197 (Multhoff et al., 1995a/b; Botzler at al., 1996; Multhoff et al., 1997). This antibody also has been found to inhibit the cytolytic activity of cells against Hsp70 expressing tumor cells (Multhoff et al., 1995b). By using Hsp70 deletion mutants that either lack the C- or the N-terminal domain the binding epitope of the mAb RPN1197 could be localized within the C-terminal substrate binding domain of Hsp70 between aa 428-618 (Botzler et al., 1998). Due to the fact that RPN1197 exhibits an inhibitory effect on

the cytolytic activity of NK cells against Hsp70 expressing tumor cells it was of interest to map the binding epitope. By peptide scanning (pepscan) of the C-terminal substrate binding domain within aa 384-618 the 8-mer peptide NLLGRFEL (aa 454-461) could be determined as the relevant recognition structure for the mAb RPN1197 (Table I a).

Table I a.

Results of the pepscan analysis of the C-terminal domain of Hsp70 protein (aa 384 – 618); 13-mer peptides with an overlap of 11 peptides were tested. The region 444 – 471 is shown; positive pepspots are marked in bold.

Positive pepspots	Sequence region aa 444 – 471 of Hsp70 protein
aa 444-471	EGERAMTKDNNLLGRFELSGIPPAPRGV
aa 444-456	EGERAMTKDNNLL
aa 446-458	---ERAMTKDNNLLGR
aa 448-461	------AMTKDNNLLGRFEL
aa 450-463	---------TKDNNLLGRFELSG
aa 452-465	----------DNNLLGRFELSGIP
aa 454-467	----------NLLGRFELSGIPPA
aa 456-469	----------LGRFELSGIPPAPR
aa 458-471	----------RFELSGIPPAPRGV

Example 2

Analysis of the immunostimulatory capacity of different Hsp70 peptides

An incubation of NK cells with low dose IL-2 (100 IU/ml) plus recombinant human Hsp70 protein (rHsp70) or the C-terminal domain of Hsp70hom has been found to increase the proliferative response of human NK cells, as compared to NK cells that had been stimulated with IL-2 only (Multhoff et al. 1999). In an effort to define the minimal immunostimulatory sequence within the C-terminal domain of Hsp70, three peptides have been synthesized. Based on the 8-mer antibody binding epitope

(NLLGRFEL) of RPN1197 mAb, a C- (GIPP) and an N-terminal (TKD) extended peptide were produced to test them in a standard ^{3}H thymidine uptake assay. The C- and N-terminal extensions were in accordance to the primary sequence of human Hsp70 (Table I b).

Table I b.

Amino acid (aa) sequences of peptides that were used for NK stimulation assays.

Origin of protein	aa	Sequence	Name (length)
Hsp70	454-461	------NLLGRFEL	NLL (8-mer)
Hsp70	454-466	------NLLGRFELSGIPP	GIPP (13-mer)
Hsp70	450-463	TKDNNLLGRFELSG	TKD (14-mer)

As an internal control the proliferative capacity of NK cells against intact recombinant Hsp70 protein (rHsp70) was investigated. A concentration of 10 µg/ml of rHsp70 has been defined as an optimal stimulatory dose, previously (loc. sit). With respect to the molecular weight of the different peptides the concentration which is equivalent to 10 µg full length Hsp70 protein (72 kDa) was calculated as 0.2 µg/ml for TKD (1563 Da) and GIPP (1452 Da) and as 0.1 µg/ml for NLL (942 Da). With respect to these results all peptides were tested at a concentration range of 0.02 µg up to 8 µg/ml. As shown in Figure 1 the 8-mer peptide NLL and the C-terminal extended peptide GIPP did not stimulate the proliferative capacity of NK cells at any of the tested peptide concentrations. However, the N-terminal extended 14-mer peptide TKD exhibits a comparable immunostimulatory capacity like full length Hsp70 protein at a concentration range between 0.2 up to 2 µg/ml. This peptide concentration is comparable to a concentration of 10 –100 µg/ml of full length Hsp70 protein.

The proliferative response of T cells derived from the same donors was also tested against the peptides plus low dose IL-2 (100 IU/ml). As expected none of the three

peptides NLL, GIPP or TKD stimulates T cell growth at any of the tested concentrations (data not shown). However, experiments are in progress investigating the immune response of T cells against complexes consisting of rHsp70 protein and the Hsp70 peptides NLL, GIPP, TKD.

Since TKD appears to stimulate the proliferative activity of NK cells the question arises whether this peptide, similar to Hsp70 protein, also stimulates the cytolytic activity. The role of Hsp70 membrane expression as a tumor specific recognition structure for the cytolytic activity of NK cells was demonstrated with HLA identical colon carcinoma sublines CX+ and CX- that differ profoundly with respect to their capacity to express Hsp70 on the plasma membrane (14). In the present study the cytolytic response of NK cells stimulated for 4 days either with IL-2 alone (100 IU/ml) or with IL-2 plus NLL or IL-2 with TKD peptide (2 µg/ml) were compared. A phenotypic characterization of the effector cells was performed directly before stimulation and on day 4 after stimulation. The results are shown in the legend of Figure 2. IL-2 plus TKD stimulated NK cells exhibited a significantly enhanced lytic activity against Hsp70 expressing CX+ tumor cells compared to NK cells that were stimulated either with IL-2 alone or with IL-2 plus NLL peptide. However, no significant differences in lysis of CX- tumor cells was observed after stimulation with either of the peptides. This finding indicates that the immunostimulatory effects of the 14-mer TKD peptide on NK cells is Hsp70 specific.

Example 3

Comparison of NK stimulatory and non-stimulatory Hsp70 peptides and protein sequences

A comparison of the 14-mer stimulatory peptide sequence (TKD) of Hsp70 (indicated in bold) with the adequate region of Hsp70hom reveals one conservative amino acid exchange at position 462 from serin (S) to threonin (T) as shown in Table II.

Tabl II.

Comparison of the amino acid (aa) sequences of peptides and proteins with NK-

stimulatory and non-stimulatory capacity.

origin	aa	sequence	NK cell stimulation
Hsp70 (peptide)	454-461	- - - - - - N L L G R F E L (8mer peptide)	no
Hsp70 (peptide)	454-466	- - - - - - N L L G R F E L S G I P P (13mer peptide)	no
Hsp70 (peptide)	450-463	T K D N N L L G R F E L S G (14mer peptide)	yes
Hsp70 (peptide)	441-463	E G E R A M T K D N N L L G R F E L S G (20mer peptide)	yes
Hsp70hom (complete protein)	450-463	T K D N N L L G R F E L T G (complete protein)	yes
Hsc70 (complete protein)	450-463	T K D N N L L G K F E L T G (complete protein)	no
Dank (complete protein)	447-460	A A D N K S L G Q F N L D G (complete protein)	no

Since, both proteins Hsp70 and Hsp70hom and the 14-mer peptide TKD are able to stimulate NK cells the aa at position 462 seems to be not relevant for the stimulation of NK cells. In contrast, the conservative aa exchange at position 458 from arginine (R) to lysine (K) between Hsp70 and Hsc70 might be of importance since only Hsp70 but not Hsc70 is able to activate NK cells. Furthermore, this aa exchange might be responsible for the specificity of the Hsp70 specific antibody RPN1197. This antibody is known to react with Hsp70 but does not cross-react with Hsc70. The only aa difference of Hsp70 and Hsc70 within the 8-mer antibody binding epitope (aa 454-460) is the exchange at position 458 from arginine (R) to lysine (K).

In a further experiment that was scheduled as outlined above, only peptides were tested for their capacity to stimulate NK cells, see Table III, below. All these experiments allow the conclusion that amino acids TKDN in positions 450 to 453, R in position 458 and S in position 462 are crucial for the effectiveness of the peptide.

Tabl III

Comparison of the amino acid (aa) sequences only of peptides (but not proteins) with NK-stimulatory and non-stimulatory capacity. The immunostimulatory capacity of the different peptides was determined in ^{3}H thymidine uptake assays and ^{51}Cr release assays. Amino acid exchanges to the 14-mer stimulatory peptide are indicated in bold.

origin	aa	sequence	NK cell stimulation
Hsp70 (NLL)	454-461	------NLLGRFEL	no
Hsp70 (GIPP)	454-466	------NLLGRFELSGIPP	no
Hsp70 (TKD)	450-463	TKDNNLLGRFELSG	yes
Hsp70 (TKD12)	450-461	TKDNNLLGRFEL	no
Hsp70	445-463	GERAMTKDNNLLGRFELSG	yes
Hsp70hom (HOM)	450-463	TKDNNLLGRFELTG	low
Hsc70 (Hsc70)	450-463	TKDNNLLGKFELTG	no
Dnak (DNAK)	447-460	AADNKSLGQFNLDG	no

Conclusions

In summary these data provide evidence that not only full length Hsp70 protein or the C-terminal domain of Hsp70hom is able to stimulate proliferation and the cytolytic activity of NK cells against Hsp70 expressing tumor cells but also a 14-mer peptide which is part of the C-terminal domain of Hsp70. The sequence of the 14-mer peptide is an N-terminal extension of the 8-mer binding epitope of the Hsp70

specific antibody RPN1197. This mAb not only detects plasma membrane bound Hsp70 but also has been found to inhibit NK-mediated lysis against Hsp70-positive tumor cells. This is the first report that NK cells can be stimulated by a 14-mer peptide derived from the C-terminal region of Hsp70.

Recently, the existence of HSP specific receptors has been shown for antigen-presenting cells by internalization experiments of gold-labeled HSP and by confocal microscopy (Arnold-Schild et al., 1999; Asea et al., 2000). Functionally, we demonstrated that Hsp70 specific receptors also might exist on NK cells (Multhoff et al., 1999). Investigations are in progress to answer the question whether the14-mer peptide derived from the C-terminal domain of Hsp70 physically interacts with the Hsp70 specific receptor on NK cells.

Materials and Methods

Epitope mapping analysis

The monoclonal antibody RPN1197 reacts only with the inducible 72 kDa HSP, and is of similar reactivity to the antibody reported by Welch and Suhan (1986). Its specificity has been confirmed by immunoprecipitation of the 72 kDa protein from heat shocked cells. Epitope mapping analysis of mAb RPN1197 was perfomed using pepspot membranes with horseradish peroxidase conjugates and chemiluminescent luminol (Jerini Bio Tools GmbH, Berlin, Germany). Cellulose bound 13-mer peptides of the C-terminal domain of Hsp70 (aa 384-618) that exhibit an overlap of 11-mer peptides were used (Reineke et al., 1996).

Hsp70 and Hsp70 peptides

Human recombinant Hsp70 protein (rHsp70) was obtained from StressGen, Victoria, British Columbia, Canada (SPP-755). The 8-mer, 14-mer and 13-mer peptides NLLGRFEL (NLL), TKDNNLLGRFELS (TKD), NLLGRFELSGIPP (GIPP) were produced by the F-moc synthesis (fluorenylmethoxycarbonyl/ t-butyl-based solid-phase peptide chemistry method on SMPS 850 (Zinser Analytic) and ABI 488A (Perkin Elmer, Norwalk, Conn.) synthesizers. The purity of Hsp70 proteins and Hsp70 peptides was determined by the *Limulus* amebeocyte lysate assay (BioWhittaker, Maryland, USA).

NK cells

Briefly, monocyte depleted peripheral blood lymphocytes (PBL) were isolated from buffy coats of healthy human volunteers (Multhoff et al., 1995a). NK cells were purified by adherence selection following a modified protocol of Vujanovic (1993). T cells remain in the supernatant cell population.

FACScan analysis

Directly fluorescein-conjugated mAb ($CD3^{FITC}$/CD16/$CD56^{PE}$, Becton Dickinson, Heidelberg, Germany) were added to cell suspensions (0.1×10^6 cells), incubated for 20 min on ice, washed and analysed on a FACScan instrument (Becton Dickinson, Heidelberg, Germany). The percentage of positively stained cells was defined as the number of specifically stained, viable (propidium-iodide negative) cells minus the number of cells stained with an isotype matched control antibody on a FACScan instrument (Becton Dickinson, Heidelberg, Germany).

Tumor cell lines

The colon carcinoma sublines CX+ (>90% Hsp70 positive cells) and CX- (<20% Hsp70 positive cells) described in patent application EP -A2-084 300 5 and originally derived from CX2 colon carcinoma cells (Tumorzentrum Heidelberg, TZB 610005, Germany) that differ with respect to their capacity to express Hsp70 on their plasma membrane were cultured at 37°C, 5% CO_2 in RPMI-1640 medium (Gibco, Eggenstein, Germany) supplemented with heat-inactivated 10% FCS (Gibco) and 2 mM L-glutamine and antibiotics (penicillin/streptomycin). The cell lines were kept in culture under exponential growth conditions, and harvested with trypsin/EDTA solution. The experiments were performed between passage 10-30. All cell lines were free from mycoplasma contamination as determined by repeated testing using the 6-methylpurin desoxyribosid assay (Boehringer Mannheim, Germany).

^{3}H thymidine uptake assay

The proliferative capacity of NK and T cells against different Hsp70 peptides and Hsp70 protein (loc. sit) was determined in a standard ^{3}H thymidine uptake assay

(22). Viable cells (5 x 10^4 cells /100 µl) were seeded in 96-well flat-bottom microtiter plates (Greiner Nuertingen, Germany) in supplemented RPMI-1640 medium containing 100 IU IL-2 and Hsp70 protein (10 µg/ml) or different amounts of Hsp70 peptides ranging from 0.02 up to 10 µg/ml. As an internal control the proliferation against IL-2 alone was measured in parallel. After a 24 or 48 hour incubation period the cells were pulsed with ^{3}H thymidine (1 µCi/well) and the total uptake was measured following an 18 hour incubation period at 37°C in a liquid scintillation counter (Beckmann instruments, Munich, Germany).

Cell mediated lympholysis assay (CML)

The cytolytic activity of NK cells was monitored in a standard ^{51}Cr assay (23). Dilutions of the effector cells were incubated with ^{51}Cr-labeled (100 µCi of $Na^{51}CrO_4$, NEN-Dupont, Boston, MA) tumor target cells (3 x 10^3 cells per well) in duplicates at a final volume of 200 µl RPMI-1640 medium supplemented with 10% FCS at 37°C for 4 h in 96-well U-bottom plates (Greiner, Nuertingen, Germany). After the incubation period supernatants were collected and the radioactivity was counted in a γ-counter (Packard Instruments). The percentage of specific lysis was determined according to the equation: (experimental release – spontaneous release) / (maximum release - spontaneous release) x 100. The percentage spontaneous release was always <15% for each target cell line.

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CLAIMS

1. A peptide comprising or having the following amino acid sequence TKDNNLLGRFELXG wherein X is T or S
or a fragment or derivative thereof.

2. The peptide of claim 1 comprising or having the amino acid sequence EGERAMTKDNNLLGRFELXG wherein X is T or S
or a fragment or derivative thereof..

3. A fusion (poly)peptide comprising the peptide of claim 1 or 2.

4. A polynucleotide encoding the peptide of claim 1 or 2 or the fusion polypeptide of claim 3.

5. The polynucleotide of claim 5 which is DNA.

6. A vector comprising the DNA of claim 5.

7. A method of refining the peptide of claim 1 or 2 comprising
   (a) modeling said peptide by peptidomimetics; and
   (b) chemically synthesizing the modeled peptide.

8. A composition comprising the peptide of claim 1 or 2 or a fragment or derivative thereof, the fusion (poly)peptide of claim 3, the polynucleotide of claim 4 or 5, the vector of claim 6 or a peptide refined by the method of claim 7 and a pharmaceutically acceptable carrier and/or diluent.

9. The composition of claim 8 which is a pharmaceutical composition, medical product, medical adjuvant or vaccine.

10. A method of producing a immunostimulatory peptide comprising

(a) mutating on the DNA or amino acid level a DNA molecule encoding or peptide comprising the sequence TKDNNLLGRFELXG, wherein X is T or S, in one or more nucleotide or amino acid positions;

(b) testing the proliferative response of NK cells to stimulation with IL-2 and/or IL-18 together with the mutated peptide;

(c) comparing the proliferative response of NK cells to IL-2 and/or IL-18 together with the mutated peptide to the proliferative response to IL-2 and/or IL-18 without the mutated peptide or with a peptide having the sequence recited in (a);

(d) selecting a peptide comprising the mutated sequence or a recombinant DNA molecule coding for a peptide comprising the mutated sequence, wherein said mutated peptide displays an increase in the proliferative response as compared to IL-2 and/or IL-18 without the mutated peptide or with a peptide having the sequence recited in (a) in step (c).

11. The method of claim 10, wherein the product is refined by computer redesign and/or peptidomimetics.

12. A method for producing a pharmaceutical composition, medical product, medical adjuvant or vaccine comprising formulating the product selected by the method of claim 10 or 11 with a pharmaceutically acceptable carrier and/or diluent.

13. A method of producing a pharmaceutical composition comprising a molecule stimulating the activation of NK cells comprising the steps of

(a) modifying the peptide of claim 1 or 2 as a lead compound to achieve

(i) modified site of action, spectrum of activity, organ specificity, and/or

(ii) improved potency, and/or

(iii) decreased toxicity (improved therapeutic index), and/or

(iv) decreased side effects, and/or

(v) modified onset of therapeutic action, duration of effect, and/or

(vi) modified pharmakinetic parameters (resorption, distribution,

metabolism and excretion), and/or

(vii) modified physico-chemical parameters (solubility, hygroscopicity, color, taste, odor, stability, state), and/or

(viii) improved general specificity, organ/tissue specificity, and/or

(ix) optimized application form and route

by

(i) esterification of carboxyl groups, or

(ii) esterification of hydroxyl groups with carbon acids, or

(iii) esterification of hydroxyl groups to, e.g. phosphates, pyrophosphates or sulfates or hemi succinates, or

(iv) formation of pharmaceutically acceptable salts, or

(v) formation of pharmaceutically acceptable complexes, or

(vi) synthesis of pharmacologically active polymers, or

(vii) introduction of hydrophilic moieties, or

(viii) introduction/exchange of substituents on aromates or side chains, change of substituent pattern, or

(ix) modification by introduction of isosteric or bioisosteric moieties, or

(x) synthesis of homologous compounds, or

(xi) introduction of branched side chains, or

(xii) conversion of alkyl substituents to cyclic analogues, or

(xiii) derivatisation of hydroxyl group to ketales, acetales, or

(xiv) N-acetylation to amides, phenylcarbamates, or

(xv) synthesis of Mannich bases, imines, or

(xvi) transformation of ketones or aldehydes to Schiff's bases, oximes, acetales, ketales, enolesters, oxazolidines, thiozolidines

or combinations thereof; and

(b) formulating the product of said modification with a pharmaceutically acceptable carrier.

14. Use of the peptide of claim 1 or 2, the fusion (poly)peptide of claim 3, the polynucleotide of claim 4 or 5, the vector of claim 6 or of a peptide refined by the method of claim 7 or 11 or to be formulated into a pharmaceutical composition

produced by the method of claim 12 or 13 and a pharmaceutically acceptable carrier and/or diluent for the preparation of a pharmaceutical composition for the activation of NK cells.

15. The use of claim 14 wherein said activation comprises the activation of the proliferation of NK cells and/or the cytolytic activity of NK cells.

16. Use of the peptide of claim 1 or 2, the fusion (poly)peptide of claim 3, the polynucleotide of claim 4 or 5, the vector of claim 6 or of a peptide refined by the method of claim 7 or 11 or to be formulated into a pharmaceutical composition produced by the method of claim 12 or 13 and a pharmaceutically acceptable carrier and/or diluent for the preparation of a pharmaceutical composition for use in immunotherapy and/or for the treatment of diseases selected from carcinomas of lung, colorectum, pancreas, larynx, stomach, peripheral and central nervous system, other carcinomas, sarcomas, chronic myeloic leukemia (CML), acute myeloic leukemia (AML), acute lymphatic leukemia (ALL), non Hodgkin Lymphoma (NHL), myeloproliferative syndrome (MPS), myelodysplastic syndrome (MDS), plasmocytoma, other leukemias, other malignant diseases, wherein Hsp70 is present on the surface of malignant cells, an infection with an HI virus, other viral or bacterial infections wherein Hsp70 is present on the surface of infected cells, rheumatoid arthritis, lupus erythematodes, other autoimmune diseases, asthma bronchiale, or other inflammatory diseases.

Fig. 1a

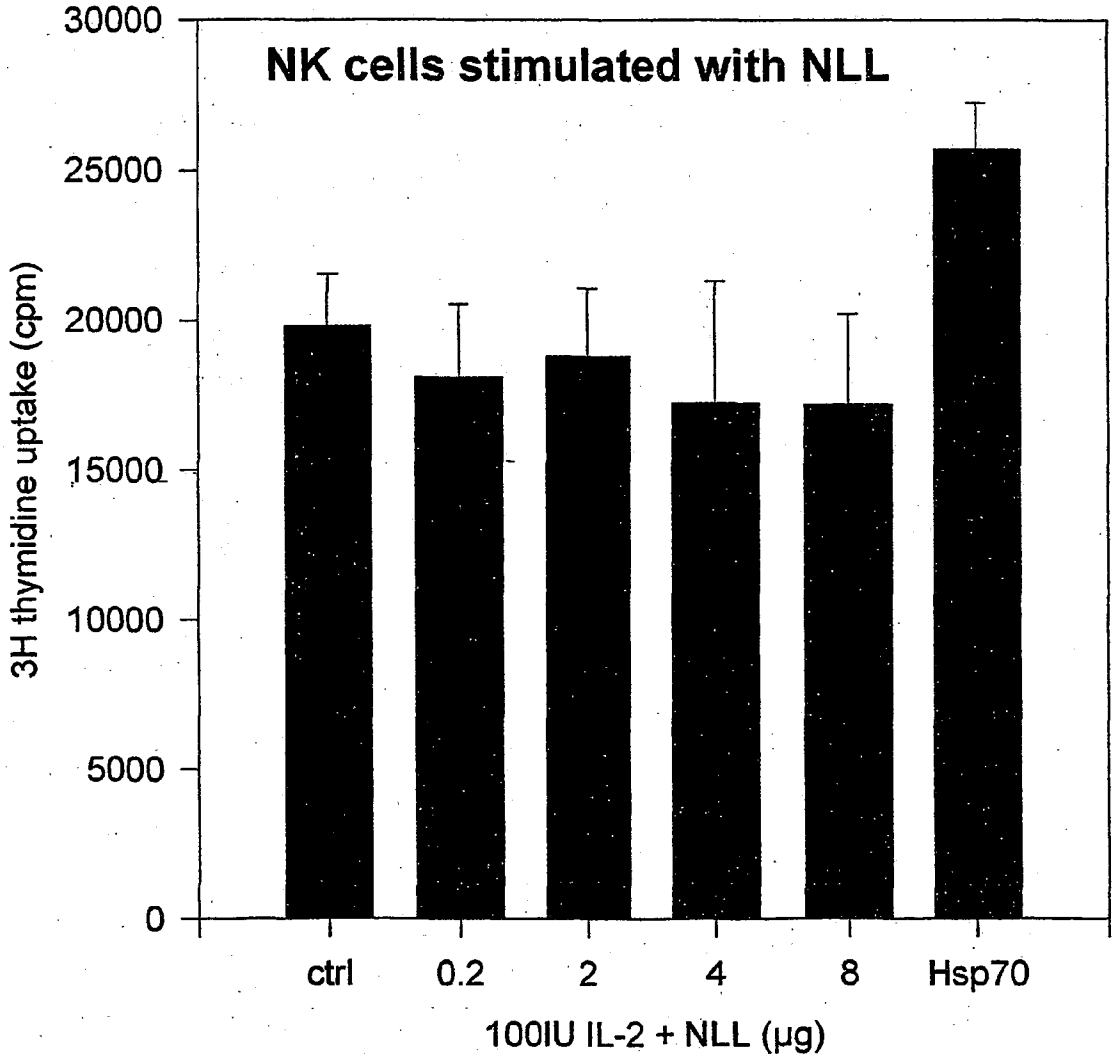
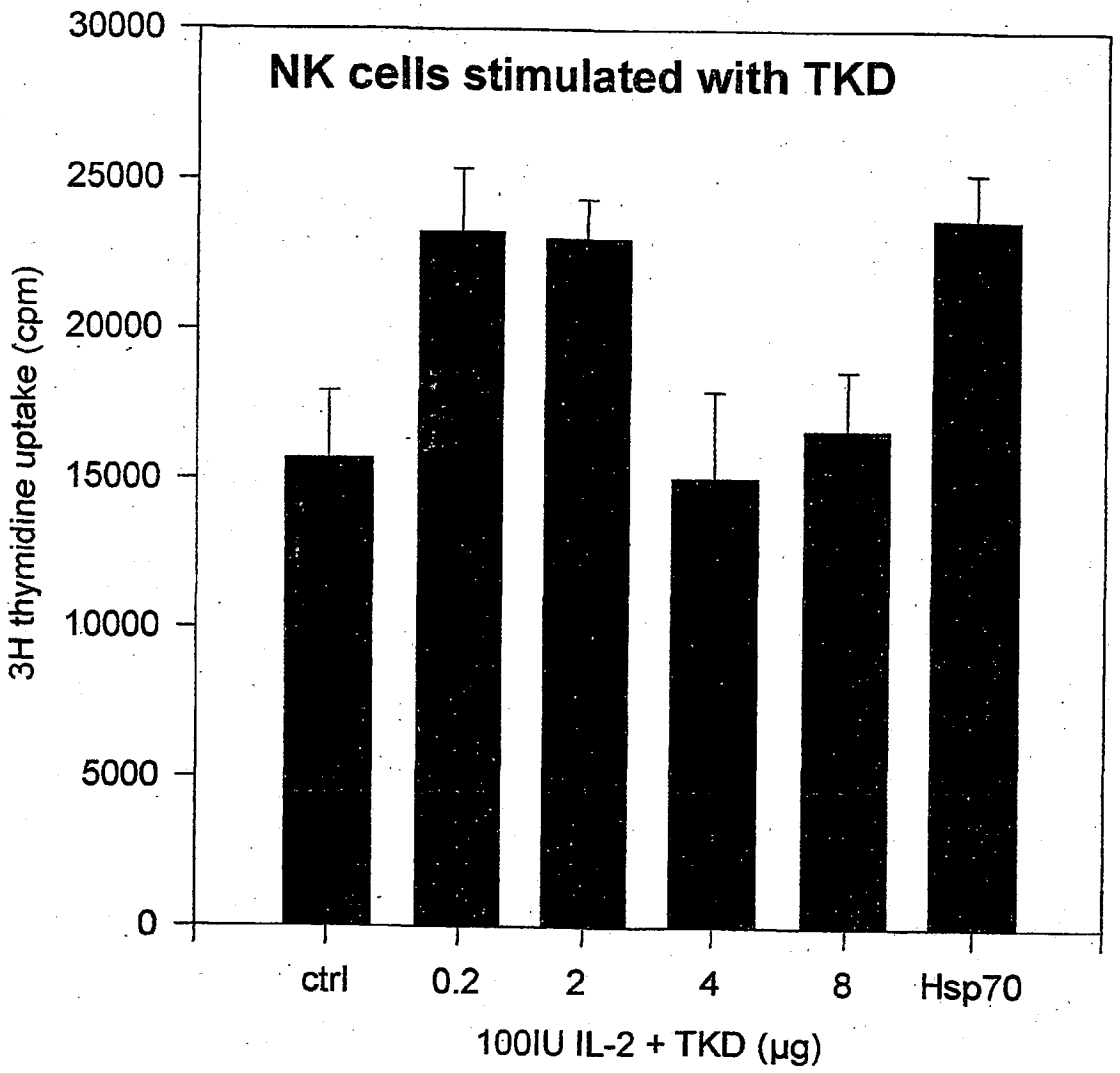

NK cells stimulated with NLL
3H thymidine uptake (cpm)
30000
25000
20000
15000
10000
5000
0
ctrl
0.2
2
4
8
Hsp70
100IU IL-2 + NLL (µg)

Fig. 1b


NK cells stimulated with TKD
30000
25000
20000
15000
10000
5000
0
3H thymidine uptake (cpm)
ctrl
0.2
2
4
8
Hsp70
100IU IL-2 + TKD (µg)

3/8

Fig. 1c

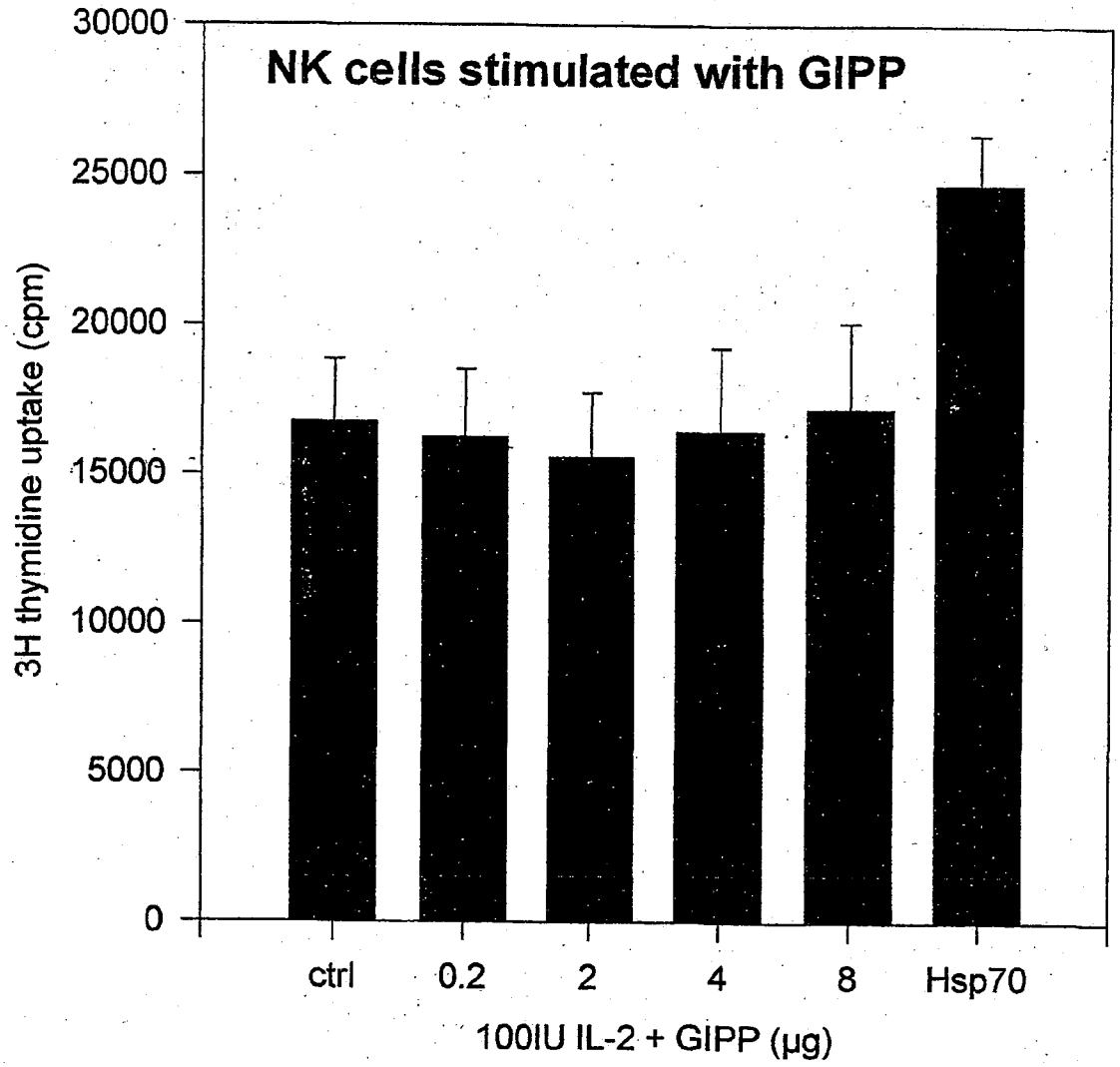

NK cells stimulated with GIPP
3H thymidine uptake (cpm)
30000
25000
20000
15000
10000
5000
0
ctrl
0.2
2
4
8
Hsp70
100IU IL-2 + GIPP (µg)

Fig. 1d

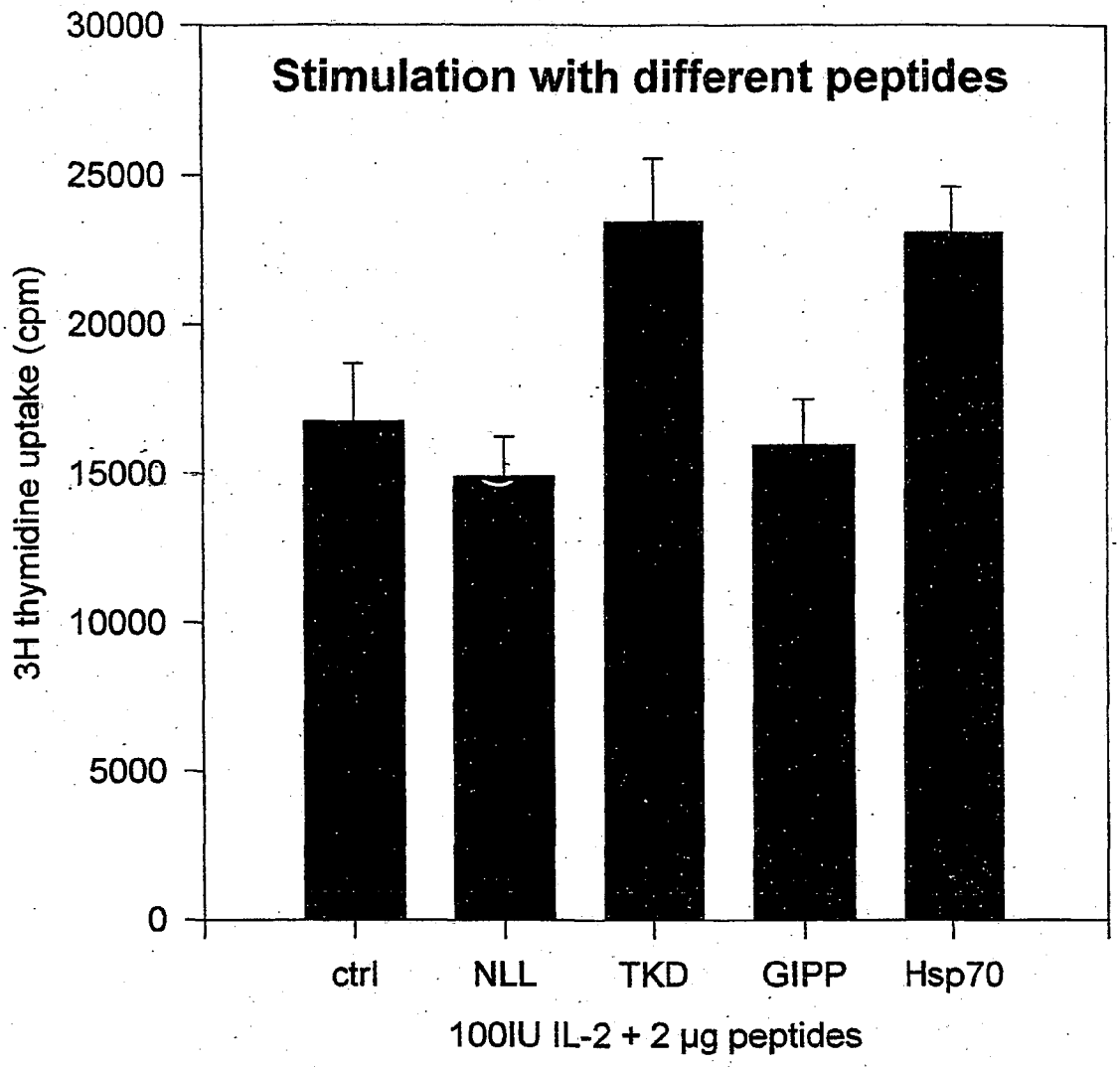

Stimulation with different peptides
3H thymidine uptake (cpm)
30000
25000
20000
15000
10000
5000
0
ctrl
NLL
TKD
GIPP
Hsp70
100IU IL-2 + 2 µg peptides

Fig. 1e

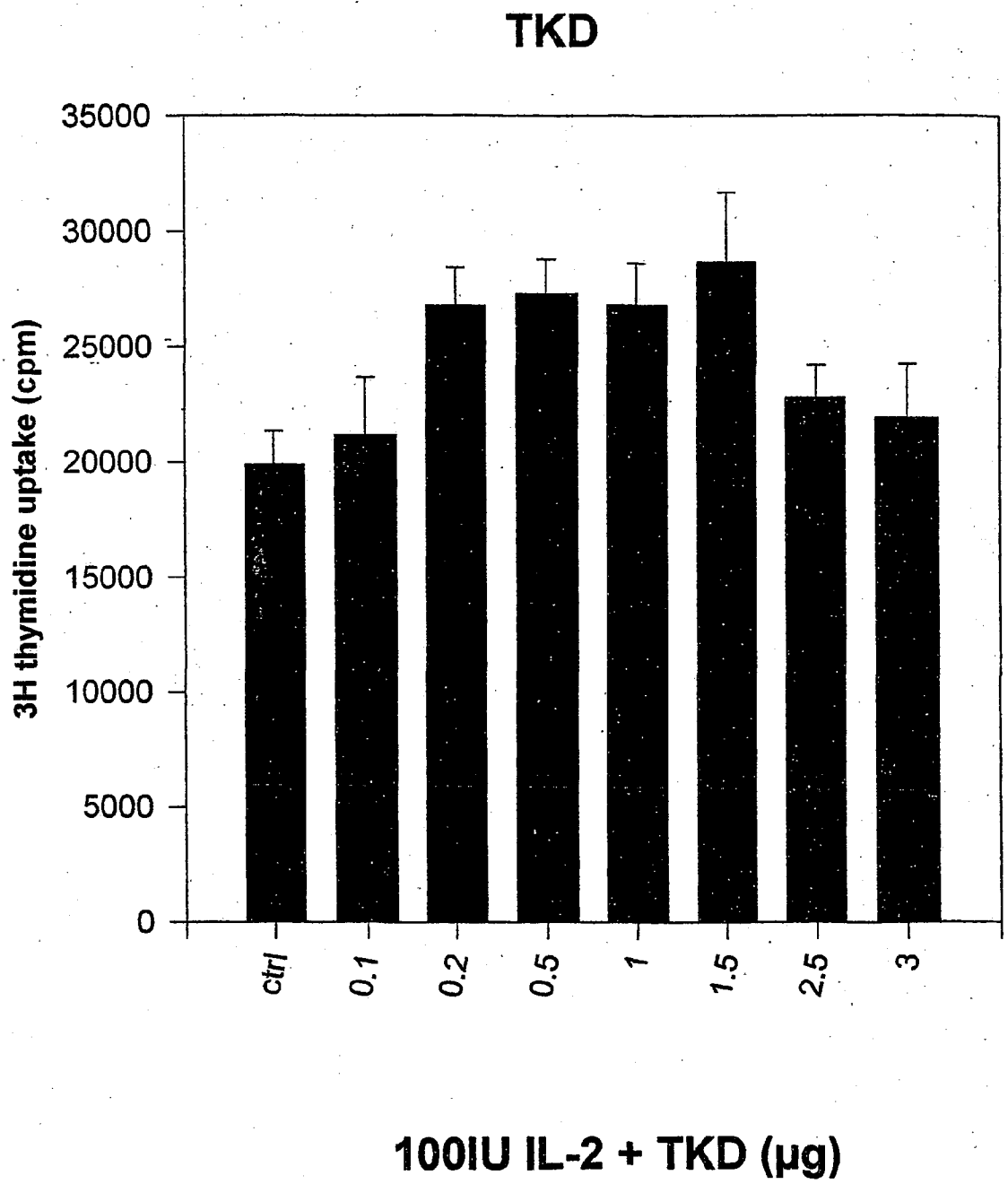

TKD
35000
30000
25000
20000
15000
10000
5000
0
3H thymidine uptake (cpm)
ctrl
0.1
0.2
0.5
1
1.5
2.5
3
100IU IL-2 + TKD (µg)

Fig. 1f

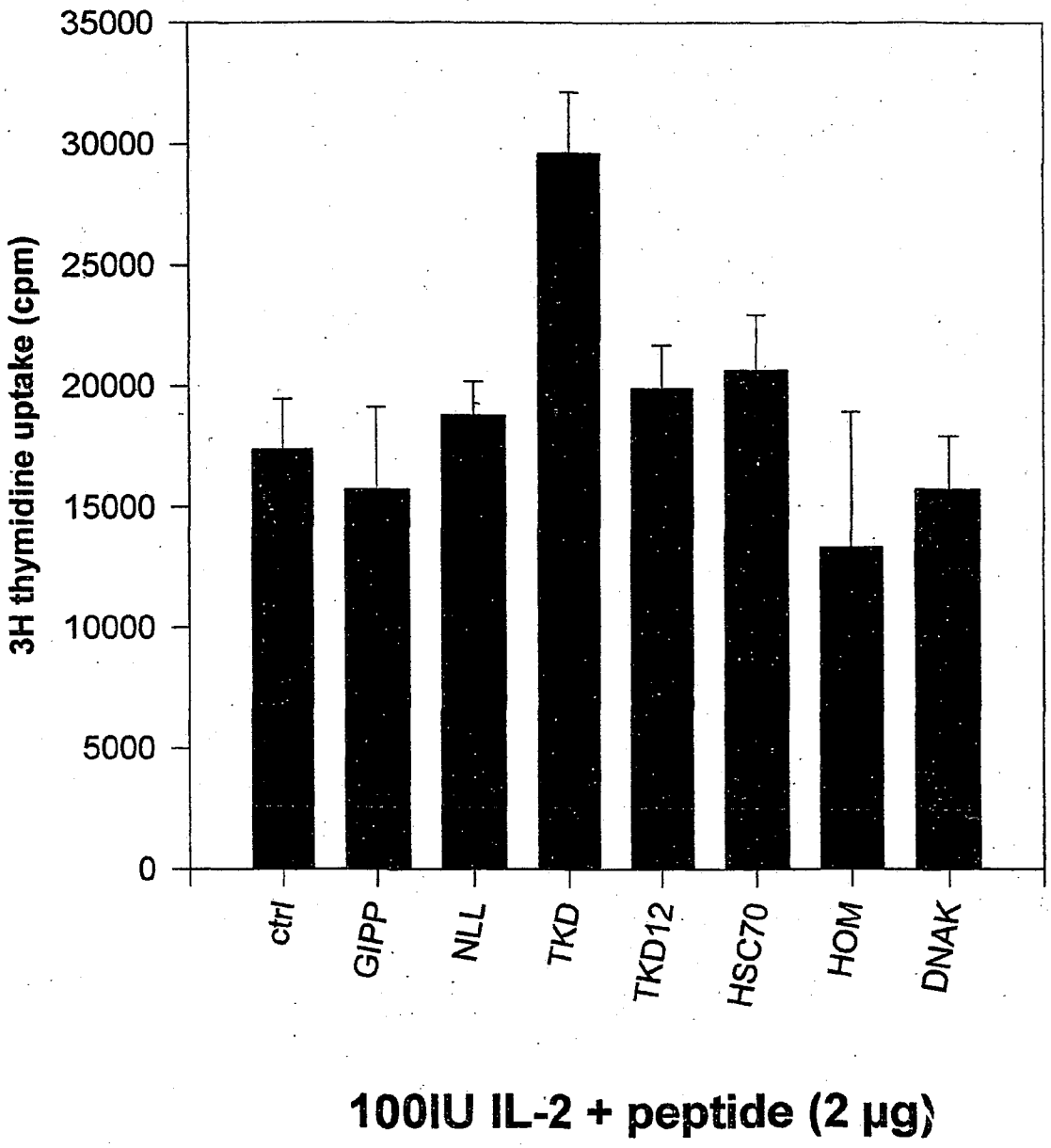

35000
30000
25000
20000
15000
10000
5000
0
3H thymidine uptake (cpm)
ctrl
GIPP
NLL
TKD
TKD12
HSC70
HOM
DNAK
100IU IL-2 + peptide (2 µg)

Fig. 2a

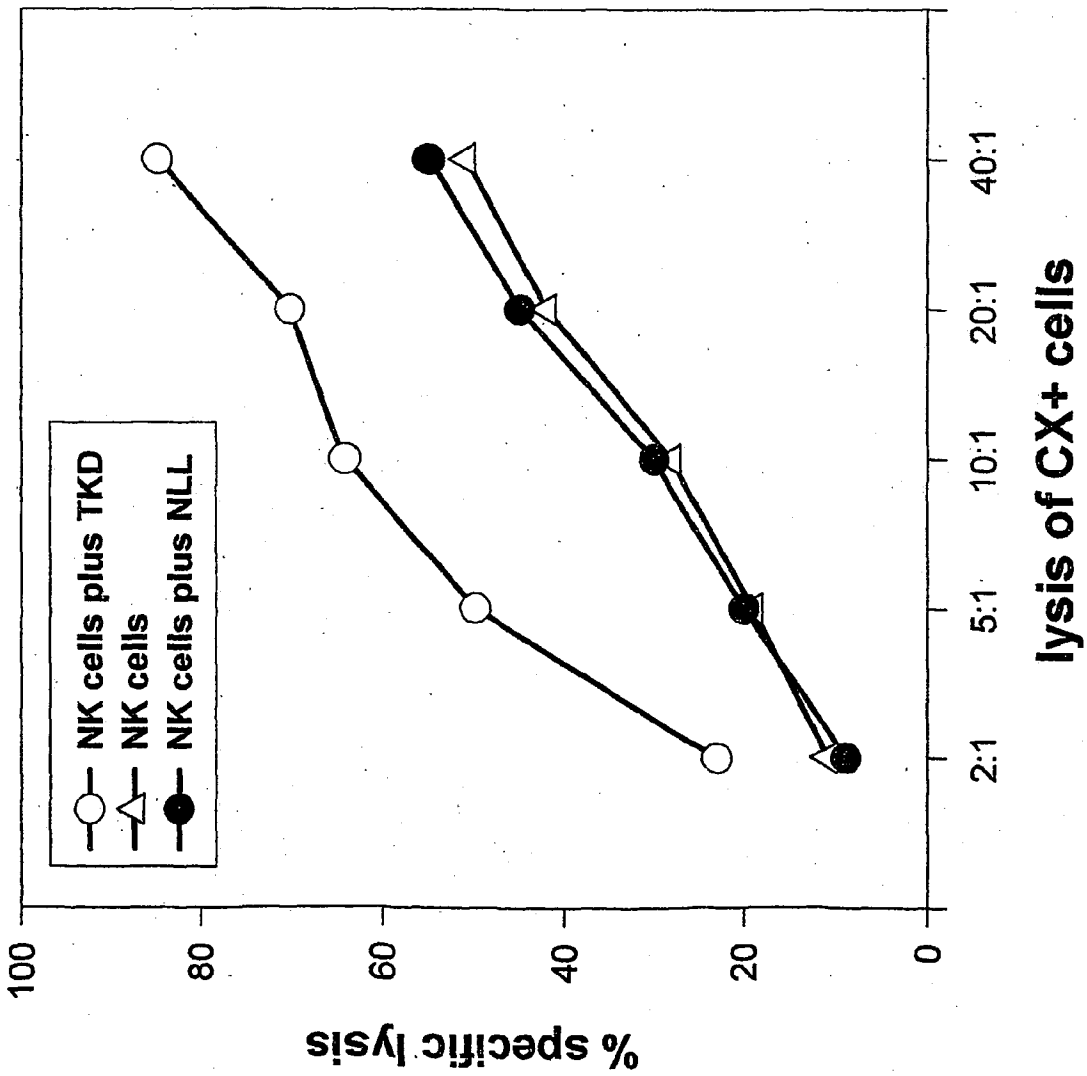
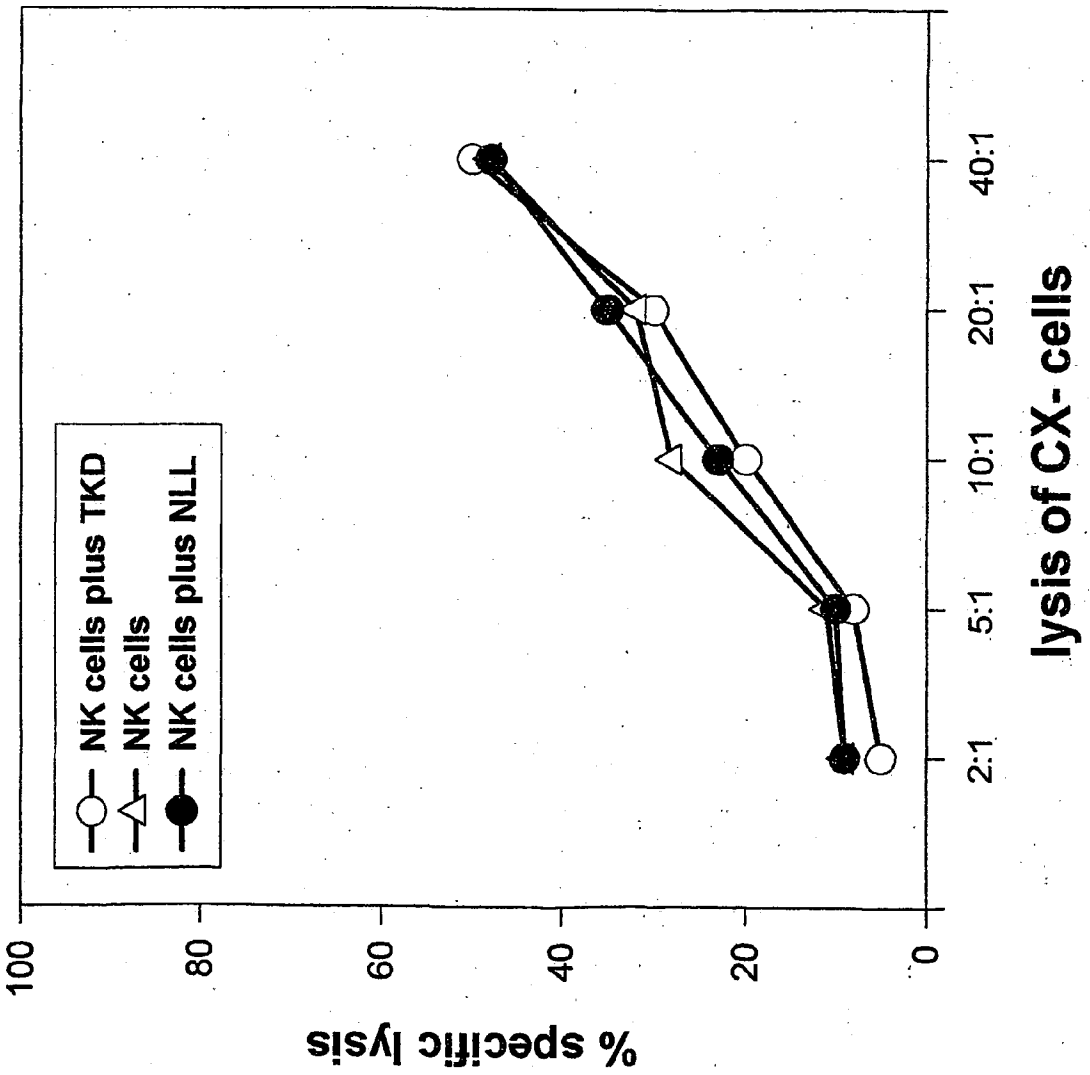

% specific lysis
100
80
60
40
20
0
2:1
5:1
10:1
20:1
40:1
lysis of CX+ cells
NK cells plus TKD
NK cells
NK cells plus NLL

Fig. 2b


NK cells plus TKD
NK cells
NK cells plus NLL
% specific lysis
0
20
40
60
80
100
2:1
5:1
10:1
20:1
40:1
lysis of CX- cells

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
E 2022 PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 01/ 10593	13/09/2001	13/09/2000
Applicant		
MULTHOFF, Gabriele		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 2 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/10593

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C07K7/08 A61K38/04 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, SEQUENCE SEARCH, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 49881 A (MULTHOFF GABRIELE) 7 October 1999 (1999-10-07) p. 4 middle paragraph page 17 -page 18	1-16
X	WO 00 31113 A (SQUIBB BRISTOL MYERS CO) 2 June 2000 (2000-06-02) p.9 , line 21 to p. 10, line 5 page 17; example 4	1-6,8,9
A	MULTHOFF G ET AL: "The role of heat shock proteins in the stimulation of an immune response" BIOLOGICAL CHEMISTRY, XX, XX, vol. 379, no. 3, March 1998 (1998-03), pages 295-300, XP002118462 ISSN: 1431-6730	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 February 2002	28/06/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stolz, B

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

[redacted]nation on patent family members

[redacted]ernational Application No
PCT/EP 01/10593

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9949881 A	07-10-1999	DE 19813760 A1	07-10-1999
		WO 9949881 A2	07-10-1999
		EP 1066050 A2	10-01-2001
WO 0031113 A	02-06-2000	AU 1731400 A	13-06-2000
		EP 1133517 A1	19-09-2001
		WO 0031113 A1	02-06-2000

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 24 July 2002 (24.07.02)	
International application No. PCT/EP01/10593	**Applicant's or agent's file reference** E 2022 PCT
International filing date (day/month/year) 13 September 2001 (13.09.01)	**Priority date** (day/month/year) 13 September 2000 (13.09.00)
Applicant MULTHOFF, Gabriele	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

12 April 2002 (12.04.02)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Farid ABBOU
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* E 2022 PCT

Box No. I TITLE OF INVENTION

An Hsp70 peptide stimulating Natural Killer (NK) cell activity and uses thereof

Box No. II APPLICANT ☒ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

MULTHOFF, Gabriele
Kirchenstraße 17c
81675 München
DE

Telephone No.

Facsimile No.

Teleprinter No.

Applicant's registration No. with the Office

State *(that is, country)* of nationality: DE

State *(that is, country)* of residence: DE

This person is applicant for the purposes of: ☒ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Vossius & Partner
Siebertstraße 4
81675 Munich
Germany

Telephone No. 089/413040

Facsimile No. 089/41304111

Teleprinter No.

Agent's registration No. with the Office

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. V DESIGNATION OF STATES *Mark the applicable check-boxes below; at least one must be marked.*

The following designations are hereby made under Rule 4.9(a):

Regional Patent

- ☒ **AP** **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **MZ** Mozambique, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **TZ** United Republic of Tanzania, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ **EA** **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH & LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, **TR** Turkey, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ **AE** United Arab Emirates
- ☒ **AG** Antigua and Barbuda
- ☒ **AL** Albania
- ☒ **AM** Armenia
- ☒ **AT** Austria
- ☒ **AU** Australia
- ☒ **AZ** Azerbaijan
- ☒ **BA** Bosnia and Herzegovina
- ☒ **BB** Barbados
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- ☒ **CR** Costa Rica
- ☒ **CU** Cuba
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- ☒ **DK** Denmark
- ☒ **DM** Dominica
- ☒ **DZ** Algeria
- ☒ **EC** Ecuador
- ☒ **EE** Estonia
- ☒ **ES** Spain
- ☒ **FI** Finland
- ☒ **GB** United Kingdom
- ☒ **GD** Grenada
- ☒ **GE** Georgia
- ☒ **GH** Ghana
- ☒ **GM** Gambia
- ☒ **HR** Croatia
- ☒ **HU** Hungary
- ☒ **ID** Indonesia
- ☒ **IL** Israel
- ☒ **IN** India
- ☒ **IS** Iceland
- ☒ **JP** Japan
- ☒ **KE** Kenya
- ☒ **KG** Kyrgyzstan
- ☒ **KP** Democratic People's Republic of Korea
- ☒ **KR** Republic of Korea
- ☒ **KZ** Kazakhstan
- ☒ **LC** Saint Lucia
- ☒ **LK** Sri Lanka
- ☒ **LR** Liberia
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- ☒ **TZ** United Republic of Tanzania
- ☒ **UA** Ukraine
- ☒ **UG** Uganda
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- ☒ **UZ** Uzbekistan
- ☒ **VN** Viet Nam
- ☒ **YU** Yugoslavia
- ☒ **ZA** South Africa
- ☒ **ZW** Zimbabwe

Check-boxes below reserved for designating States which have become party to the PCT after issuance of this sheet:

☒ PH Philippines ☐ ☐

☐ ☐ ☐

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		national application: country	regional application:* regional Office	international application: receiving Office
item (1) 13 September 2000 (13.09.00)	00 11 9933.0		EP	
item (2)				
item (3)				
item (4)				
item (5)				

☐ Further priority claims are indicated in the Supplemental Box.

The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office)* identified above as:

☐ all items ☒ item (1) ☐ item (2) ☐ item (3) ☐ item (4) ☐ item (5) ☐ other, see Supplemental Box

** Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii)):* . . . .

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA / EP

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII DECLARATIONS

The following **declarations** are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)*: Number of declarations

☐ Box No. VIII (i)	Declaration as to the identity of the inventor	:
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	:
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	:
☐ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)	:
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	:

Box No. IX CHECK LIST; LANGUAGE OF FILING

This international application **contains**:

(a) the following number of **sheets in paper form**:

request (including declaration sheets)	:	4
description (excluding sequence listing part)	:	33
claims	:	4
abstract	:	1
drawings	:	8
Sub-total number of sheets	:	50
sequence listing part of description *(actual number of sheets if filed in paper form, whether or not also filed in computer readable form; see (b) below)*	:	
Total number of sheets	:	50

(b) sequence listing part of description filed in **computer readable form**

(i) ☐ only (under Section 801(a)(i))

(ii) ☐ in addition to being filed in paper form (under Section 801(a)(ii))

Type and number of carriers (diskette, CD-ROM, CD-R or other) on which the sequence listing part is contained *(additional copies to be indicated under item 9(ii), in right column)*: .....

This international application is **accompanied by** the following item(s) *(mark the applicable check-boxes below and indicate in right column the number of each item)*:

Number of items

1. ☐ fee calculation sheet :
2. ☐ original separate power of attorney :
3. ☐ original general power of attorney :
4. ☐ copy of general power of attorney; reference number, if any: ..... :
5. ☐ statement explaining lack of signature :
6. ☐ priority document(s) identified in Box No. VI as item(s): ..... :
7. ☐ translation of international application into *(language)*: ..... :
8. ☐ separate indications concerning deposited microorganism or other biological material :
9. ☐ sequence listing in computer readable form (indicate also type and number of carriers (diskette, CD-ROM, CD-R or other))
   - (i) ☐ copy submitted for the purposes of international search under Rule 13*ter* only (and not as part of the international application) :
   - (ii) ☐ *(only where check-box (b)(i) or (b)(ii) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Rule 13*ter* :
   - (iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing part mentioned in left column :
10. ☐ other *(specify)*: ..... :

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Dr. Joachim Wachenfeld

European Patent Attorney

Vossius & Partner
Siebertstr. 4
81675 München
(Nr. 31)

For receiving Office use only

1. Date of actual receipt of the purported international application:

2. Drawings:
   ☐ received:
   ☐ not received:

3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:

4. Date of timely receipt of the required corrections under PCT Article 11(2):

5. International Searching Authority (if two or more are competent): ISA /

6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International Application No.

Date stamp of the receiving Office

Applicant's or agent's file reference: E 2022 PCT

Applicant: MULTHOFF, Gabriele

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . EUR 102.00 [T]

2. SEARCH FEE . . . . . EUR 945.00 [S]

International search to be carried out by EP

(If two or more International Searching Authorities are competent to carry out the international search, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

Where item (b) of Box No. IX applies, enter **Sub-total number of sheets** / Where item (b) of Box No. IX does not apply, enter **Total number of sheets**: 50

[b1] first 30 sheets . . . . . EUR 409.00 [b1]

[b2] 20 (number of sheets in excess of 30) x 9.00 (fee per sheet) = EUR 180.00 [b2]

[b3] additional component (only if sequence listing part of description is filed in computer readable form under Section 801(a)(i), or both in that form and on paper, under Section 801(a)(ii)):

400 x 9.00 (fee per sheet) = EUR [b3]

Add amounts entered at b1, b2 and b3 and enter total at B . . . . EUR 589.00 [B]

Designation Fees

The international application contains all designations.

6 (number of designation fees payable *(maximum 6)*) x 88.00 (amount of designation fee) = EUR 528.00 [D]

Add amounts entered at B and D and enter total at I . . . . . EUR 1,117.00 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . EUR 30.00 [P]

5. TOTAL FEES PAYABLE . . . . . EUR 2,194.00 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☒ authorization to charge deposit account (see below) ☐ postal money order ☐ cash ☐ coupons

☐ cheque ☐ bank draft ☐ revenue stamps ☐ other *(specify)*:

AUTHORIZATION TO CHARGE (OR CREDIT) DEPOSIT ACCOUNT

(This mode of payment may not be available at all receiving Offices)

Receiving Office: RO/ EPO

Deposit Account No.: 2800.0321

Date: 13 October 2001

Name: Dr. Joachim Wachenfeld

Signature:

☒ Authorization to charge the total fees indicated above.

☒ *(This check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* Authorization to charge any deficiency or credit any overpayment in the total fees indicated above.

☒ Authorization to charge the fee for priority document.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
VOSSIUS & PARTNER
Siebertstrasse 4
81675 München
GERMANY

EINGEGANGEN
Vossius & Partner
28. Juni 2002
Frist bearb.: 28 8. 28 7.

Applicant's or agent's file reference E 2022 PCT	Date of mailing *(day/month/year)* 28/06/2002 **FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/EP 01/10593	International filing date *(day/month/year)* 13/09/2001
Applicant MULTHOFF, Gabriele	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Heike Zoglauer

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

INTE TIONAL SEARCH REPORT

International Application No
PCT/EP 01/10593

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C07K7/08 A61K38/04 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, SEQUENCE SEARCH, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 49881 A (MULTHOFF GABRIELE) 7 October 1999 (1999-10-07) p. 4 middle paragraph page 17 -page 18	1-16
X	WO 00 31113 A (SQUIBB BRISTOL MYERS CO) 2 June 2000 (2000-06-02) p.9 , line 21 to p. 10, line 5 page 17; example 4	1-6,8,9
A	MULTHOFF G ET AL: "The role of heat shock proteins in the stimulation of an immune response" BIOLOGICAL CHEMISTRY, XX, XX, vol. 379, no. 3, March 1998 (1998-03), pages 295-300, XP002118462 ISSN: 1431-6730	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 February 2002	28/06/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stolz, B

1

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
E 2022 PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 01/10593	13/09/2001	13/09/2000
Applicant		
MULTHOFF, Gabriele		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 2 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 01/10593

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9949881 A	07-10-1999	DE 19813760 A1	07-10-1999
		WO 9949881 A2	07-10-1999
		EP 1066050 A2	10-01-2001
WO 0031113 A	02-06-2000	AU 1731400 A	13-06-2000
		EP 1133517 A1	19-09-2001
		WO 0031113 A1	02-06-2000

VOSSIUS & PARTNER

Patentanwälte

Vossius & Partner POB 86 07 67 81634 München Germany

To the
European Patent Office
Munich

PATENTANWÄLTE
EUROPEAN PATENT ATTORNEYS
EUROPEAN TRADEMARK ATTORNEYS
Dr. VOLKER VOSSIUS, Dipl.-Chem.
(bis 1992; danach in anderer Kanzlei)
Dr. PAUL TAUCHNER, Dipl.-Chem.
Dr. DIETER HEUNEMANN, Dipl.-Phys.
Dr. PETER A. RAUH, Dipl.-Chem.
Dr. GERHARD HERMANN, Dipl.-Phys.
JOSEF SCHMIDT, Dipl.-Ing.
Dr. HANS-RAINER JAENICHEN, Dipl.-Biol.
Dr. ALEXA VON UEXKÜLL, M.Sc.
Dr. RUDOLF WEINBERGER, Dipl.-Chem.
Dr. WOLFGANG BUBLAK, Dipl.-Chem.
AXEL STELLBRINK, Dipl.-Ing.
Dr. JOACHIM WACHENFELD, (Biol.)
Dr. FRIEDERIKE STOLZENBURG, Dipl.-Biol.
RAINER VIKTOR, Dipl.-Ing.
EUROPEAN PATENT ATTORNEYS
Dr. RENATE BARTH, Dipl.-Chem.
Dr. URSULA ENGLBRECHT, Dipl.-Chem.
Dr. PETER EINMAYR, Dipl.-Chem.
RECHTSANWÄLTE
HELGA TREMMEL
Dr. JOHANN PITZ
BARBARA GUGGENMOS, Dipl.-Chem.
Dr. THURE SCHUBERT
SIMONE SCHÄFER

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+49-89-41 30 44 00 (G 3)
(Marken - Trademarks)
E-MAIL: info@vossiusandpartner.com
HOMEPAGE: www.vossiusandpartner.com

PCT/EP01/10593
MULTHOFF, Gabriele
Our Ref.: E 2022 PCT

December 11, 2001
Wa/elh

This is in response to the Invitation to correct defects in the international application issued by the European Patent Office on November 20, 2001.

As requested in Annex A, please find enclosed duly executed authorization.

As regards Annex C, please find enclosed new Figures 1/8 to 8/8, in triplicate which should meet the requirements of the PCT.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosures:
Authorization
Figures 1/8 to 8/8, in triplicate

PCT

POWER OF ATTORNEY

(for an international application filed under the Patent Cooperation Treaty)

(PCT Rule 90.4)

The undersigned applicant(s) *(Names should be indicated as they appear in the request)*:

MULTHOFF, Gabriele
Kirchenstraße 17c
81675 München
DE

hereby appoints (appoint) the following person as: ☒ agent ☐ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Vossius & Partner
Sieberstr. 4
81675 Munich
DE

(No. 31)

to represent the undersigned before

☒ all the competent International Authorities
☐ the International Searching Authority only
☐ the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: An Hsp70 peptide stimulating Natural Killer (NK) cell activity and uses thereof

Applicant's or agent's file reference: E 2022 PCT

International application number (if already available): PCT/EP01/10592

filed with the following Office EPO as receiving Office
and to make or receive payments on behalf of the undersigned

Signature of the applicant(s) *(where there are several applicants each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power)*:

Gabriele Multhoff

Date: 29.11.01

Form PCT/Model of power of attorney (for a given international application) (July 1992)

Fig. 1a

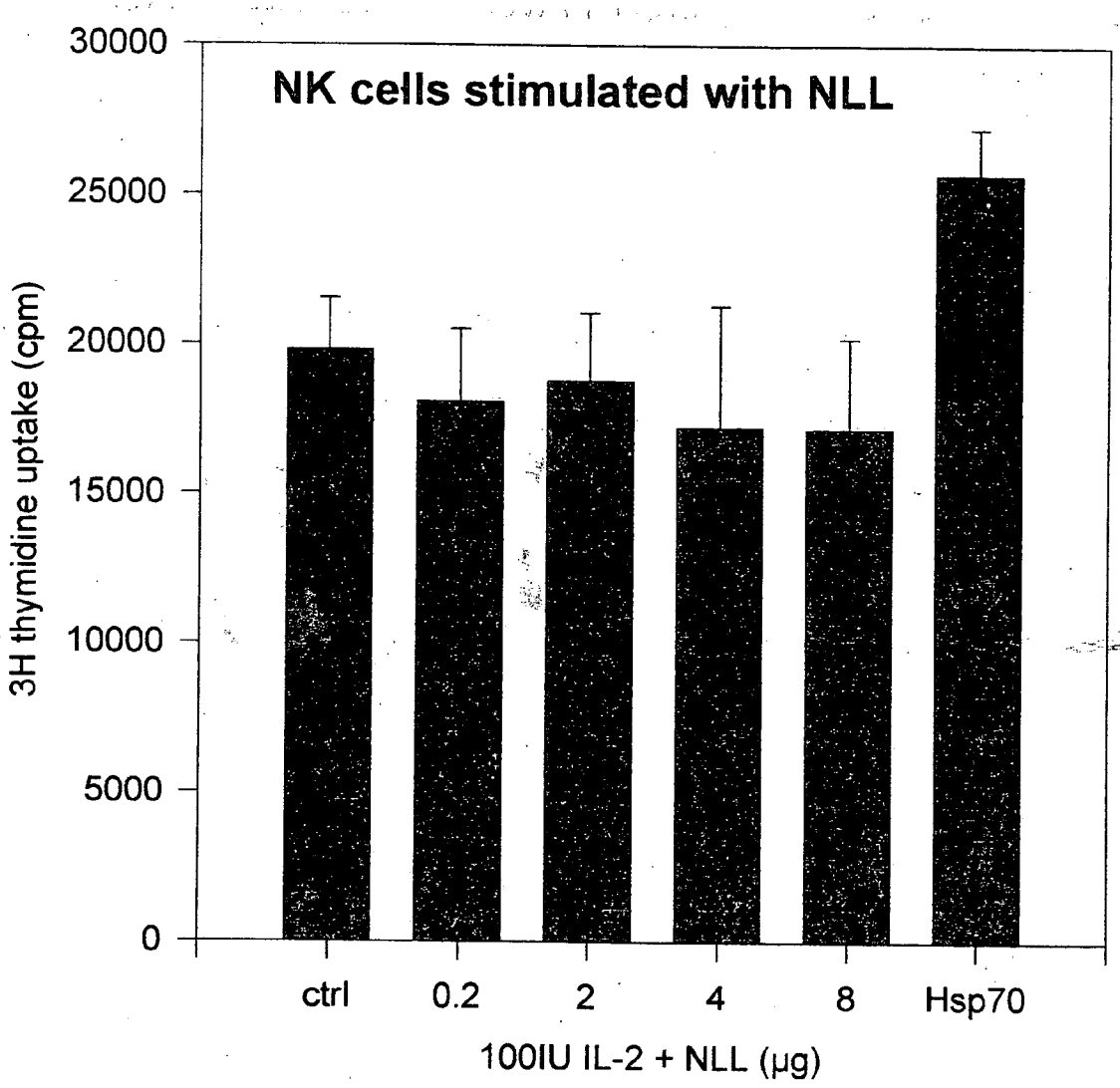

NK cells stimulated with NLL
3H thymidine uptake (cpm)
30000
25000
20000
15000
10000
5000
0
ctrl
0.2
2
4
8
Hsp70
100IU IL-2 + NLL (µg)

Fig. 1b

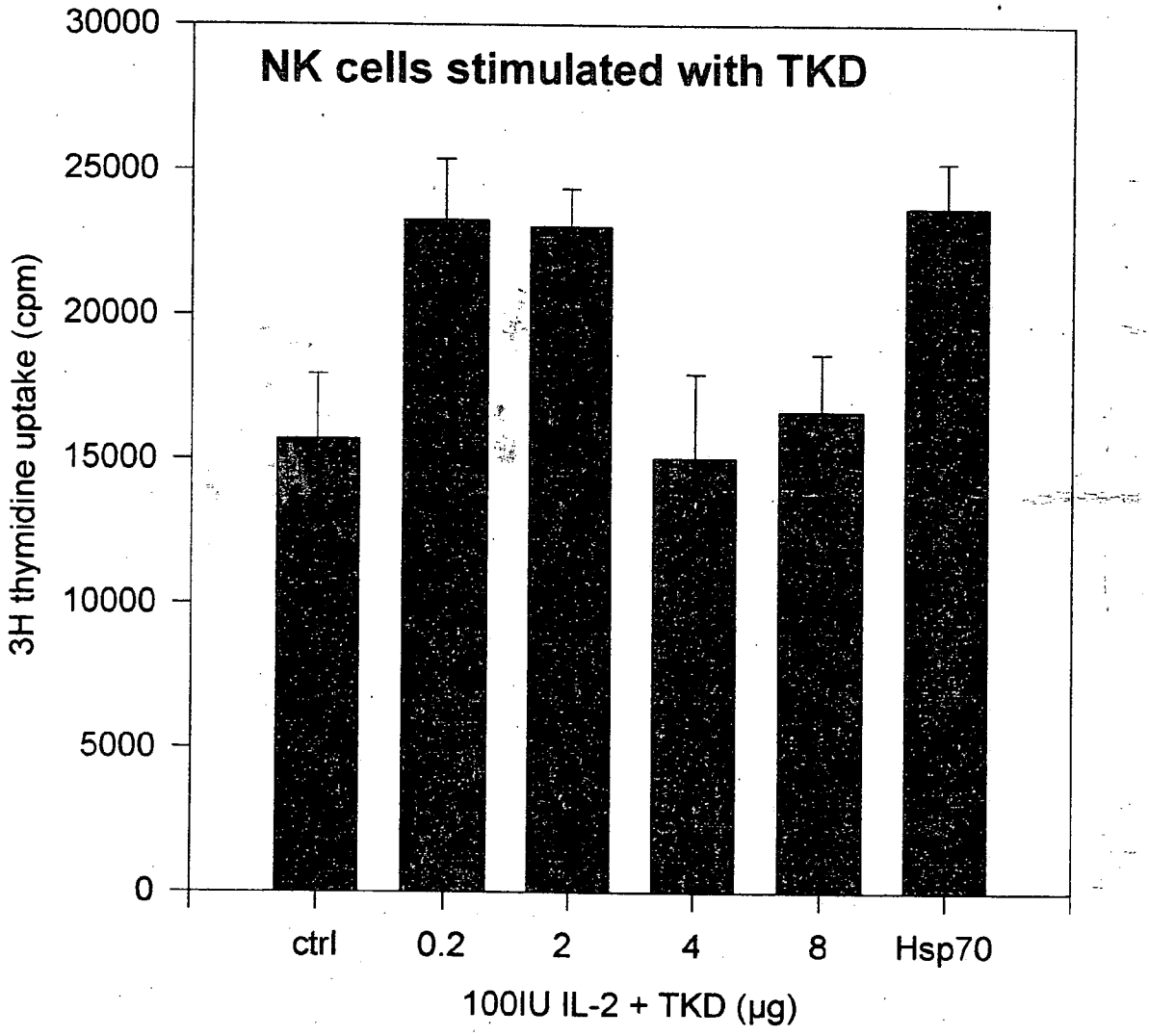

NK cells stimulated with TKD
30000
25000
20000
15000
10000
5000
0
3H thymidine uptake (cpm)
ctrl
0.2
2
4
8
Hsp70
100IU IL-2 + TKD (µg)

Fig. 1c

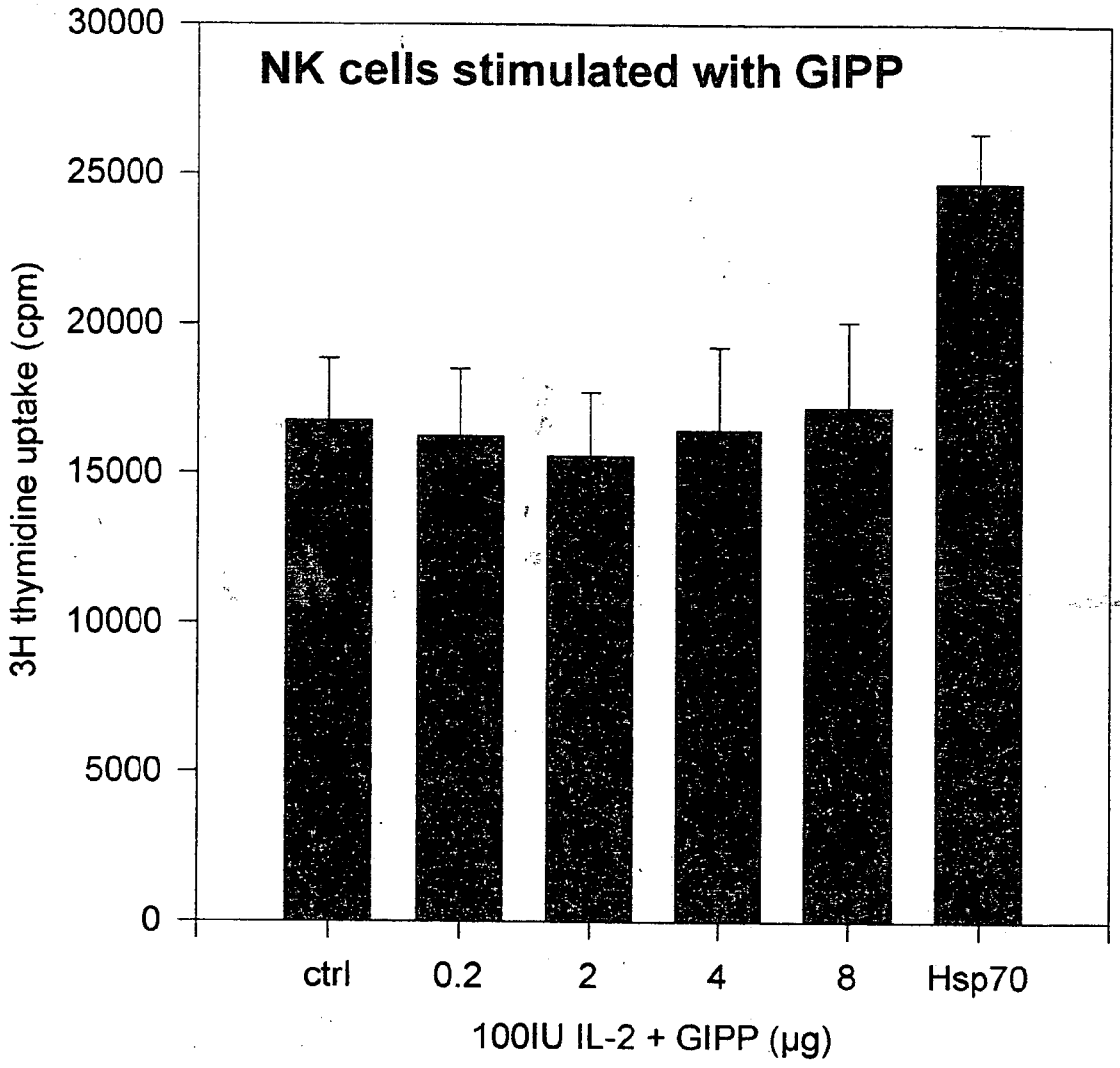

NK cells stimulated with GIPP
3H thymidine uptake (cpm)
30000
25000
20000
15000
10000
5000
0
ctrl
0.2
2
4
8
Hsp70
100IU IL-2 + GIPP (µg)

Fig. 1d

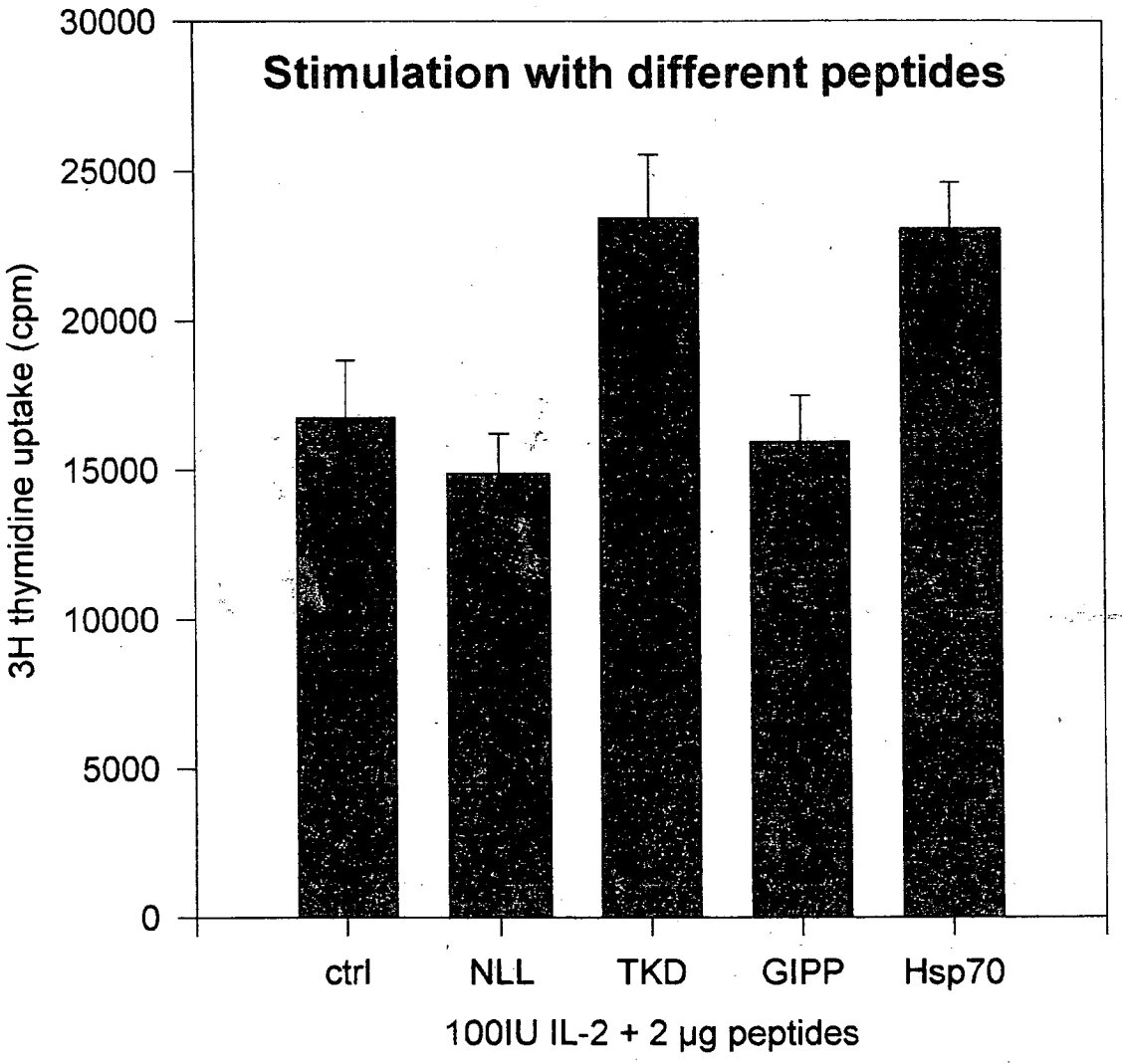

Stimulation with different peptides
3H thymidine uptake (cpm)
30000
25000
20000
15000
10000
5000
0
ctrl
NLL
TKD
GIPP
Hsp70
100IU IL-2 + 2 µg peptides

Fig. 1e

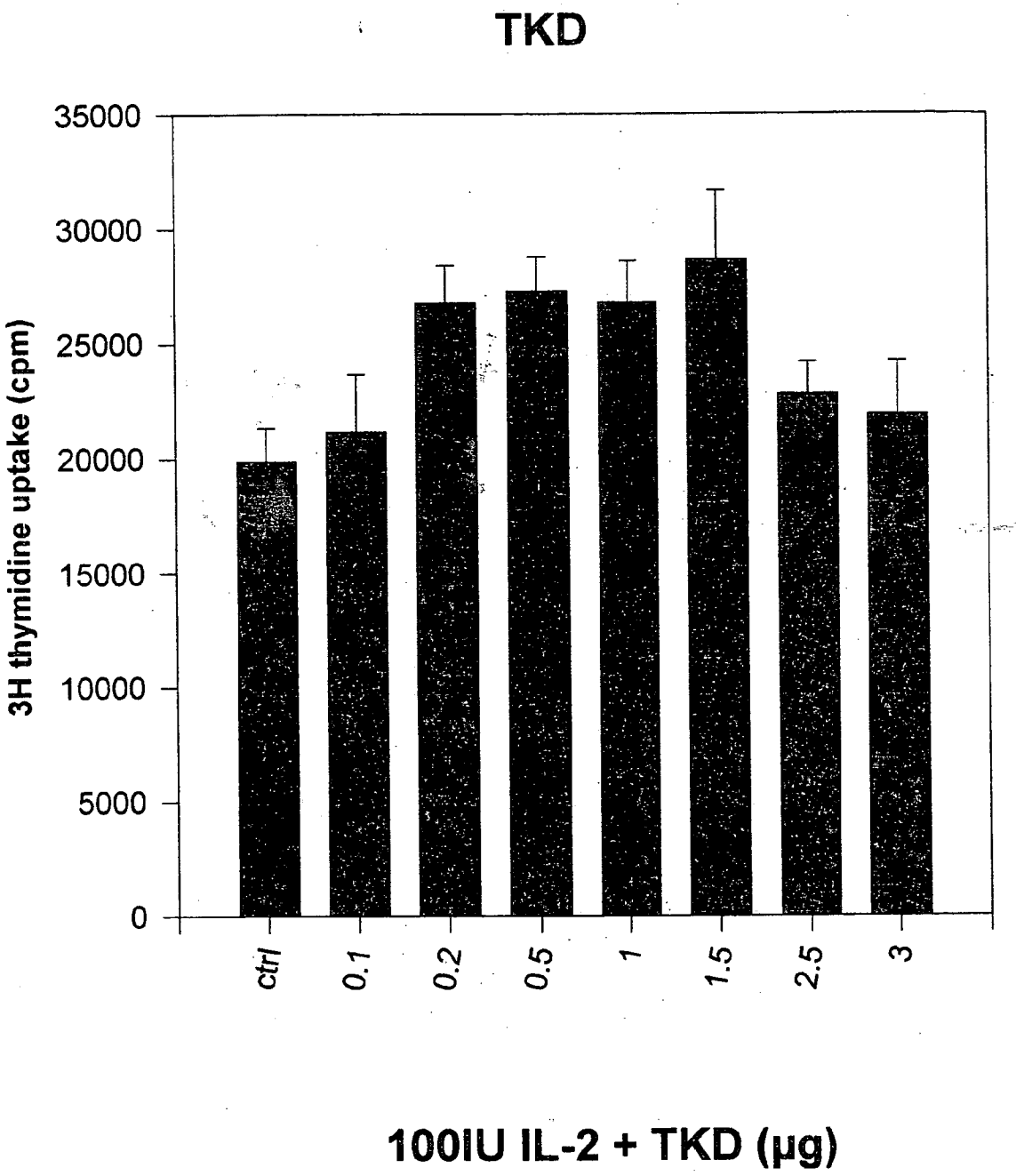

TKD
35000
30000
25000
20000
15000
10000
5000
0
3H thymidine uptake (cpm)
ctrl
0.1
0.2
0.5
1
1.5
2.5
3
100IU IL-2 + TKD (µg)

Fig. 1f

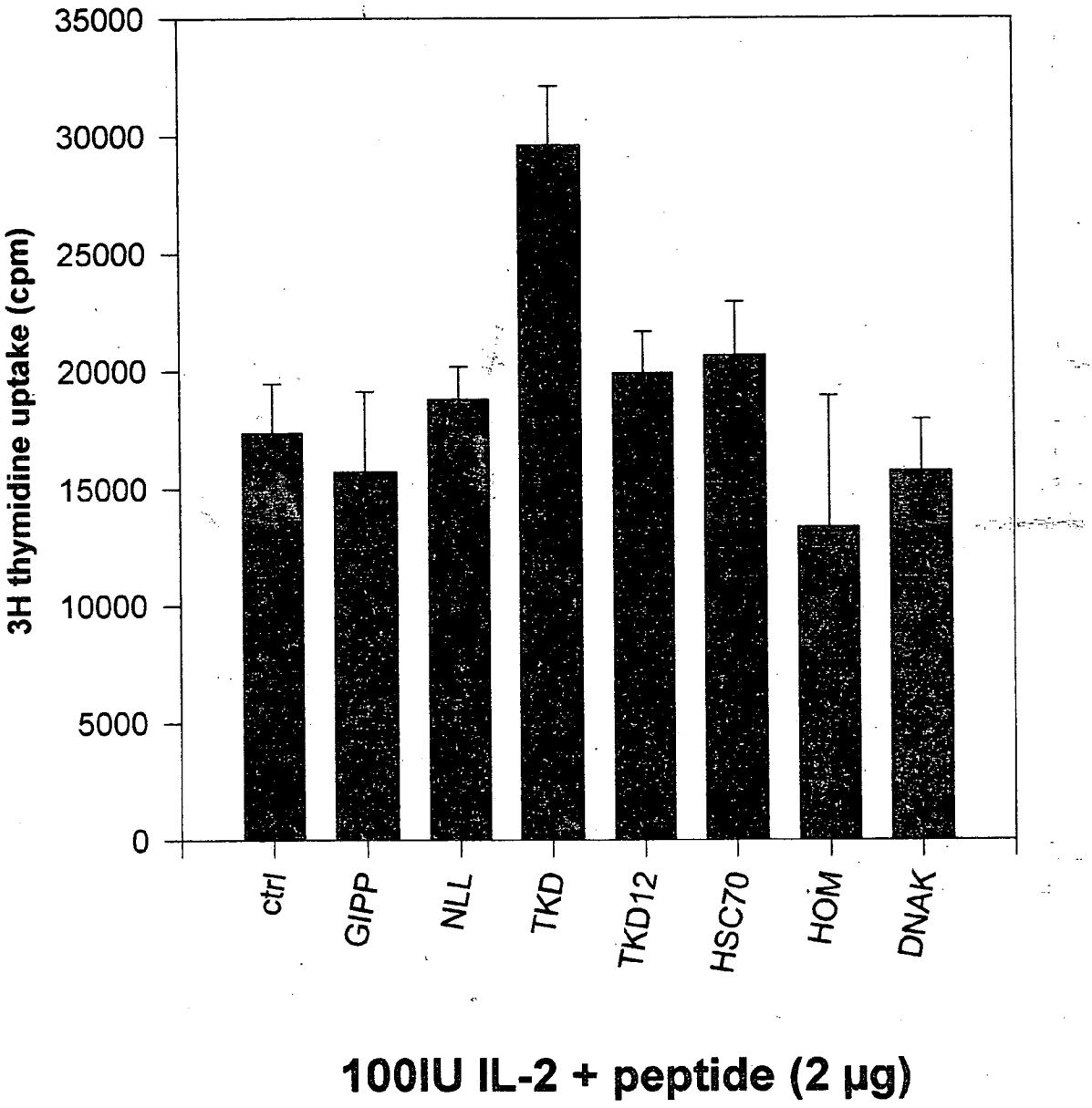

35000
30000
25000
20000
15000
10000
5000
0
3H thymidine uptake (cpm)
ctrl
GIPP
NLL
TKD
TKD12
HSC70
HOM
DNAK
100IU IL-2 + peptide (2 µg)

Fig. 2a

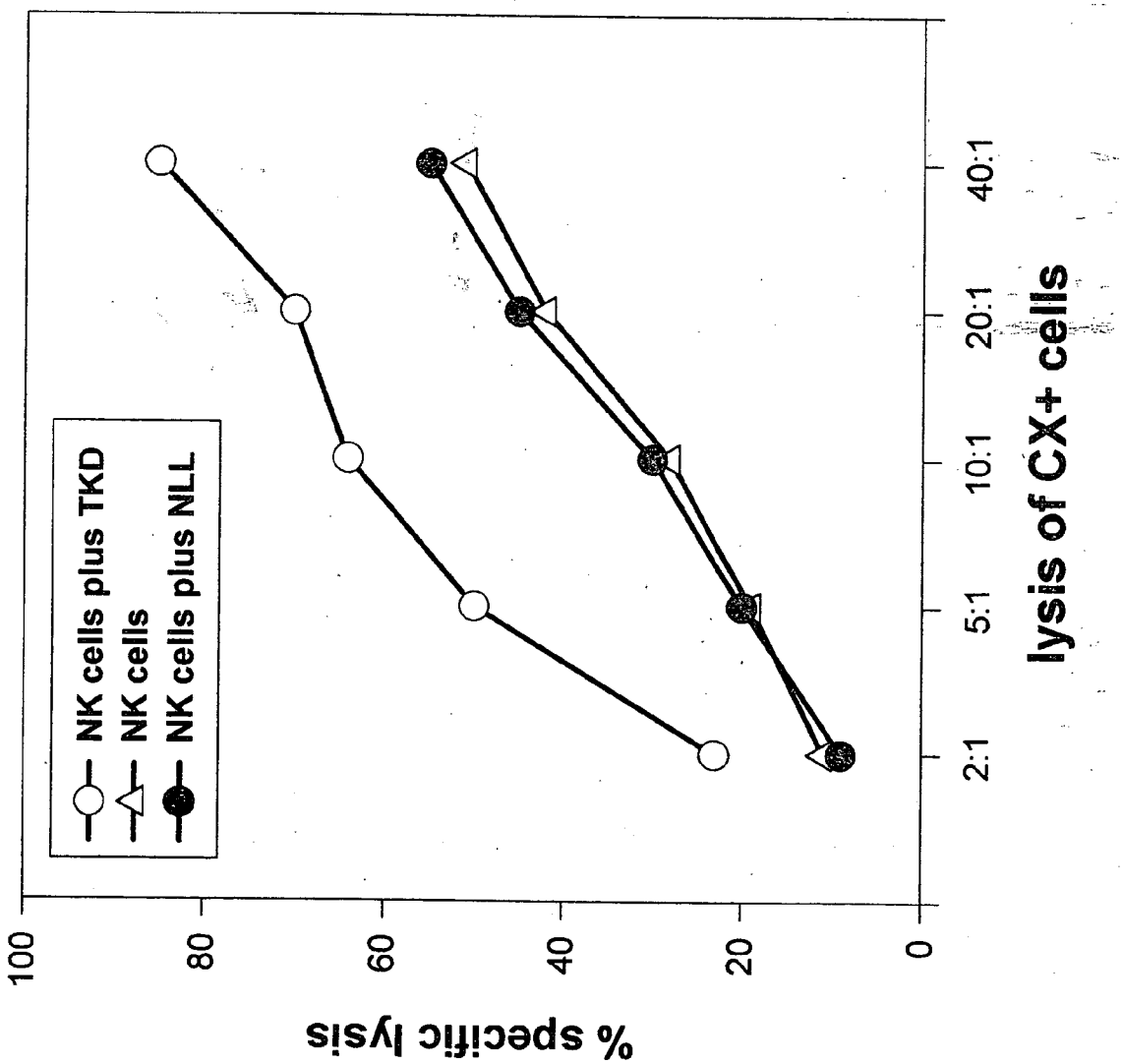
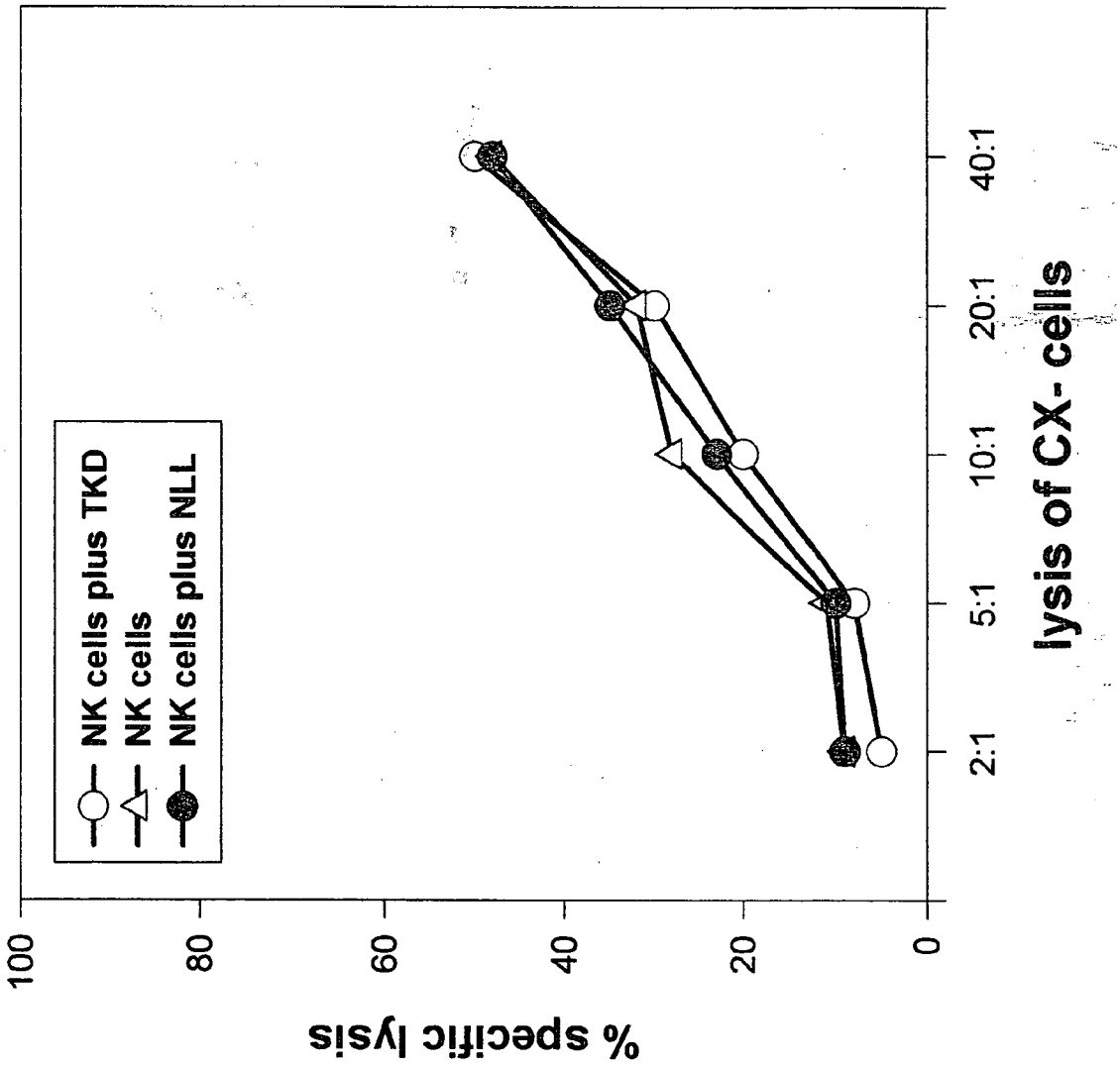

NK cells plus TKD
NK cells
NK cells plus NLL
% specific lysis
100
80
60
40
20
0
2:1
5:1
10:1
20:1
40:1
lysis of CX+ cells

Fig. 2b


NK cells plus TKD
NK cells
NK cells plus NLL
% specific lysis
100
80
60
40
20
0
2:1
5:1
10:1
20:1
40:1
lysis of CX- cells

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 March 2002 (21.03.2002)

PCT

(10) International Publication Number
WO 02/022656 A3

(51) International Patent Classification[7]: C07K 14/47, 7/08, A61K 38/04, C12N 15/11

(21) International Application Number: PCT/EP01/10593

(22) International Filing Date:
13 September 2001 (13.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
00119933.0 13 September 2000 (13.09.2000) EP

(71) Applicant and
(72) Inventor: **MULTHOFF, Gabriele** [DE/DE]; Kirchenstrasse 17c, 81675 München (DE).

(74) Agent: **VOSSIUS & PARTNER**; Siebertstrasse 4, 81675 Munich (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
26 September 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/022656 A3

(54) Title: AN HSP70 PEPTIDE STIMULATING NATURAL KILLER (NK) CELL ACTIVITY AND USES THEREOF

(57) Abstract: The present invention relates to an immunostimulatory peptide derived from an Hsp70 protein and peptides comprising said immunostimulatory peptide. Furthermore the present invention pertains to polynucleotides encoding said peptide, vectors comprising said polynucleotides, fusion (poly)peptides comprising said peptide and compositions comprising said peptide. In addition the present invention relates to the use of said peptide, polynucleotide, vector or fusion (poly)peptide, for the preparation of pharmaceutical compositions for the treatment of diseases and for the stimulation of natural killer cell (NK cell) activity.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/10593

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C07K7/08 A61K38/04 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, SEQUENCE SEARCH, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 49881 A (MULTHOFF GABRIELE) 7 October 1999 (1999-10-07) p. 4 middle paragraph page 17 -page 18	1-16
X	WO 00 31113 A (SQUIBB BRISTOL MYERS CO) 2 June 2000 (2000-06-02) p.9 , line 21 to p. 10, line 5 page 17; example 4	1-6,8,9
A	MULTHOFF G ET AL: "The role of heat shock proteins in the stimulation of an immune response" BIOLOGICAL CHEMISTRY, XX, XX, vol. 379, no. 3, March 1998 (1998-03), pages 295-300, XP002118462 ISSN: 1431-6730	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 February 2002	28/06/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stolz, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/10593

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9949881	A	07-10-1999	DE	19813760 A1	07-10-1999
			WO	9949881 A2	07-10-1999
			EP	1066050 A2	10-01-2001
WO 0031113	A	02-06-2000	AU	1731400 A	13-06-2000
			EP	1133517 A1	19-09-2001
			WO	0031113 A1	02-06-2000

The demand must be filed directly with the competent International Preliminary Examining Authority, or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority mat. be indicated by the applicant oil the line below:

IPEA/EP

PCT

DEMAND

CHAPTER II

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: E 2022 PCT

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/EP01/10593	13 September 2001 (13/09/2001)	13 September 2000 (13/09/2000)

Title of invention:
An Hsp70 peptide stimulating Natural Killer (NK) cell activity and uses thereof

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name, for a legal entity; full official designation. The address must include postal code and name of country.)*

MULTHOFF, Gabriele
Kirchenstrasse 17c
81675 München
DE

Telephone No.:

Facsimile No.:

Teleprinter No.:

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DE	DE

Name and address: *(Family name followed by given name, for a legal entity; full official designation. The address must include postal code and name of country.)*

State (that is, country) of nationality:	State *(that is, country)* of residence:

Name and address: *(Family name followed by given name, for a legal entity; full official designation. The address must include postal code and name of country.)*

State (that is, country) of nationality:	State *(that is, country)* of residence:

☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/EP01/10593

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name, for a legal entity; full official designation. The address must include postal code and name of country.)*

Vossius & Partner
Siebertstrasse 4 No. 31
81675 Munich

Telephone No.: 089 / 41 30 40

Facsimile No.: 089 / 41 304-111

Teleprinter No.:

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination **to start on the basis of:**

☒ the international application as originally filed

the description ☒ as originally filed
☐ as amended under Article 34

the claims ☒ as originally filed
☐ as amended under Article 19 (together with any accompanying statement)
☐ as amended under Article 34

the drawings ☒ as originally filed
☐ as amended under Article 34

2. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. ☐ The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This checkbox may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments *of* the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: **English**

☒ which is the language in which the international application was filed.
☐ which is the language of a translation furnished for the purposes of international search.
☐ which is the language of publication of the international application.
☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**

International application No.
PCT/EP01/10593

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ nucleotide and or amino acid sequence listing in computer readable form
6. ☐ other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

Dr. Joachim Wachenfeld
European Patent Attorney

For International Preliminary Examining Authority use only

1.	Date of actual receipt of DEMAND:	
2.	Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):	
3. ☐	The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply.	☐ The applicant has been informed accordingly.
4. ☐	The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.	
5. ☐	Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.	

For International Bureau use only

Demand received from IPEA on:

PCT

FEE CALCULATION SHEET

CHAPTER II

Annex to the Demand for international preliminary examination

International application No. PCT/EP01/10593	For International Preliminary Examining Authority use only
Applicant's or agent's file reference E 2022 PCT	Date stamp of the IPEA

Applicant

MULTHOFF, Gabriele

Calculation of prescribed fees

1. Preliminary examination fee.....	EUR	1,530.00	P
2. Handling fee (Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so en-titled, the amount to be entered at H is 25% of the handling fee.).....	EUR	159.00	H
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box.....	EUR	1,689.00	TOTAL

Mode of Payment

☒	authorization to charge deposit account with the IPEA (see below)	☐	cash
☐	cheque	☐	revenue stamps
☐	postal money order	☐	coupons
☐	bank draft	☐	other *(specify)*:

Deposit Account Authorization *(this mode of payment may not be available at all IPEAs)*

The IPEA/EP ☒ is hereby authorized to charge the total fees indicated above to my deposit account.

☒ *(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

2800.0321	09/04/2002	Dr. Joachim Waehenfeld European Patent Attorney
Deposit Account Number	Date (day/month/year)	Signature

See Notes to the fee calculation sheet

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:

VOSSIUS & PARTNER
Siebertstrasse 4
81675 München
ALLEMAGNE

EINGEGANGEN
Vossius & Partner
06. Aug. 2002
Frist bearb.: 5.9.02 G
29.8.

WRITTEN OPINION

(PCT Rule 66)

Applicant's or agent's file reference E 2022 PCT	Date of mailing *(day/month/year)* 05/08/2002
	REPLY DUE within 1 / 00 months/days from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP 01/ 10593	13/09/2001	13/09/2000

International Patent Classification (IPC) or both national classification and IPC

C07K7/00

Applicant

MULTHOFF, Gabriele

1. This written opinion is the first drawn up by this International Preliminary Examining Authority.
2. This opinion contains indications relating to the following items:

- I [X] Basis of the opinion
- II [ ] Priority
- III [ ] Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV [ ] Lack of unity of invention
- V [X] Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI [ ] Certain documents cited
- VII [ ] Certain defects in the international application
- VIII [ ] Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4*bis*.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 13/01/2003

Name and mailing address of the IPEA/	Authorized officer Examiner
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Formalities officer (incl. extension of time limits) Tel. (+49-89) 2399 2828

EUROPÄISCHES PATENTAMT · EUROPEAN PATENT OFFICE · OFFICE EUROPÉEN DES BREVETS

I. Basis of the opinion

1. The basis of this written opinion is the application as originally filed.

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability

1. In light of the documents cited in the international search report, it is considered that the invention as defined in at least some of the claims does not appear to meet the criteria mentioned in Article 33(1) PCT, i.e. does not appear to be novel and/or to involve an inventive step (see international search report, in particular the documents cited X and/or Y and corresponding claims references).

2. If amendments are filed, the applicant should comply with the requirements of Rule 66.8 PCT and indicate the basis of the amendments in the documents of the application as originally filed (Article 34 (2) (b) PCT) otherwise these amendments may not be taken into consideration for the establishment of the international preliminary examination report. The attention of the applicant is drawn to the fact that if the application contains an unnecessary plurality of independent claims, no examination of any of the claims will be carried out.

NB: Should the applicant decide to request detailed substantive examination, then an international preliminary examination report will normally be established directly. Exceptionally the examiner may draw up a second written opinion, should this be explicitly requested.

VOSSIUS & PARTNER
Patentanwälte

Vossius & Partner POB 86 07 67 81634 Munich Germany

European Patent Office
Erhardtstr. 27

80298 M Ü N C H E N

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(bis 1992; danach in anderer Kanzlei)
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PCT/EP01/10593
Dr. Gabriele Multhoff
Our Ref.: E2022 PCT

September 3, 2002
WA/DU/MEZ

This is in response to the Written Opinion pursuant to Rule 66 PCT issued by the EPO on August 5, 2002 in connection with the above identified patent application.

1. Novelty (Article 33(1) and Rule 66.2(a)(ii) PCT)

1.1 WO 99/49882 (Multhoff Gabriele)

The IPEA has assessed WO 99/49881 as being novelty destroying for subject matter of claims 1 to 16.

WO 99/49881 describes the use of Hsp70 protein of fragments thereof to activate NK cells and pharmaceuticals, medical products or medical adjuvants containing an Hsp70 protein or fragments thereof or activated NK cells. Said fragments are characterized as fragments of the C-terminus of the Hsp70 protein. The C-terminus itself is further specified as the region comprising amino acid residue 384 to 641 of the human Hsp70.

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Siebertstrasse 4
81675 Munich
Germany

POSTAL ADDRESS:
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TEL.: +49-89-41 30 40
FAX: +49-89-41 30 41 11
+49-89-41 30 44 00 (Trademarks)

EMAIL: info@vossiusandpartner.com
HOMEPAGE: www.vossiusandpartner.com

Partnerschaftsregister Amtsgericht München PR 89

In contrast, the present application discloses peptides comprising or having the specific amino acid sequences identified in claims 1 and 2. The term "peptide" as used in the application is defined on page 3 of the application. Accordingly, the term relates to peptides comprising or having "amino acid sequences comprising 30 or less amino acids". Thus, the present application discloses for the first time amino stimulatory peptides derived from Hsp70 proteins characterized by said particular amino acid sequences. Moreover, peptides comprising said amino stimulatory peptides and having a maximum length of 30 amino acids are disclosed.

Consequently, the present application is novel over WO 99/49881.

1.2 WO 00/31113 (Bristol-Myers Squibb Co.)

The IPEA has assessed WO 00/31113 as being relevant for the discussion of novelty of subject-matter of claims 1 to 6, 8 and 9.

WO 00/31113 describes "a carrier for the delivery of molecules with biological function into both cellular and nuclear compartments"; see abstract of WO 00/31113. The document describes a corresponding method as follows:

> "A method for delivering a compound into a cell or cells, said method comprising associating said compound with Hsp70, or a fragment of Hsp70, to form an Hsp70 complex, and providing said Hsp70 complex to said cell of the environment surrounding said cell so that said Hsp70 complex is imported into the cell or cells." (see claim 1, emphasis added)

Thus, an essential technical feature of the recited Hsp70 or the fragment of the Hsp70 is the ability of said protein or fragment thereof to form a complex. This Hsp70 complex also represents an essential technical feature of the pharmaceutical composition according to independent claim 9.

In contrast the present invention relates to an immuno stimulatory peptide comprising or having a particular identified amino acid sequence; see section 1.1, supra. Said polypeptide is neither described to form a complex nor is it

essential for the effect of the peptide described in the present application that said peptide forms a complex.

Consequently, the peptide of the present invention is different from the Hsp70 protein or fragment of the Hsp70 protein, capable of forming a complex, described in WO 00/31113. Thus, subject-matter of the described present application is novel over WO 00/31113.

2. Request

With the above explanations it is submitted that the subject-matter of the present invention will be acknowledged as novel over the documents cited as X-documents in the International Search Report.

Therefore, it is requested that the objections raised under Article 33(1) and Rule 66.2(a)(ii) PCT are withdrawn.

Dr. Joachim Wachenfeld
European Patent Attorney

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

VOSSIUS & PARTNER
Siebertstrasse 4
D-81675 München
ALLEMAGNE

EINGEGANGEN
Vossius & Partner
10. Dez. 2002
Frist
bearb.:

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	09.12.2002
Applicant's or agent's file reference E 2022 PCT	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP01/10593	13/09/2001	13/09/2000

Applicant
MULTHOFF, Gabriele

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

For the purpose of deciding whether the claimed invention is patentable or not, the elected Offices may apply criteria additional to or different from the criteria on which the international preliminary examination report is based (see Articles 27(5), 33(5)). Additional criteria may include e.g. exemptions from patentability and the requirements of enabling disclosure and of clarity and support of claims.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Hingel, W Tel.+49 89 2399-8717

Form PCT/IPEA/416 (July 1992)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP01/10593

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-33 as originally filed ;

Claims, No.:

1-16 as originally filed

Drawings, sheets:

1/8-8/8 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	7,10-16
	No:	Claims	1-6,8,9
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-16
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations
see separate sheet

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
E 2022 PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP01/10593	13/09/2001	13/09/2000
International Patent Classification (IPC) or national classification and IPC C07K7/00		
Applicant MULTHOFF, Gabriele		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12/04/2002	09.12.2002
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Stolz, B Telephone No. +49 89 2399 8416

Form PCT/IPEA/409 (cover sheet) (January 1994)

Reasoned statement

1. The present application relates to immunostimulatory peptides derived from HSP70.

   Applicant's comments have been taken into consideration.

2. Novelty (Art. 33(2) PCT)

   The wording of the claims is not sufficiently clear to unambiguously delimit the claimed subject matter from the prior art. The claims relate to peptides comprising the specified sequences. On p. 3 of the description preferred size limitations are indicated. These limitations are however only preferred and are moreover not part of the claims. Thus also longer peptides such as the C-terminal fragments of HSP-70 described in WO99/49881 (D1) and in WO00/31113 fall under the terms of claims 1-6, 8 and 9.

3. Inventive step (Art. 33(3) PCT)

   Claims 1 to 16 lack an inventive step in view of D1 alone.
   D1 discloses an immune modulatory role of HSP-70 and its C-terminal fragment. In particular, D1 mentions Fragments of the C-terminal fragment of HSP-70 as suitable embodiments (p. 4, middle paragraph; "umfasst von der vorliegenden Anmeldung sind auch Fragmente des C-terminalen Fragmentes 384-641"; "insofern ist der Fachmann auch ohne weiteres in der Lage, Fragmente aus dem vorstehend genannten Fragment 384-641 gentechnologisch herzustellen und auf die gewünschten Aktivierungseigenschaften hin zu testen."). An even more explicit teaching can be found in the paragraph spanning pp. 17/18. There, peptides comprising a core 7mer of sequence NLLGRFE are disclosed. These peptides can be flanked by naturally occurring flanking amino acids and/or can be modified. Thus, D1 discloses all the necessary means to modify and test further HSP-70 derived peptides.
   In the absence of a demonstrated unexpected effect of the presently claimed peptides over the peptides of D1, inventive step cannot be acknowledged.